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FEDERICO II

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dei Materiali e della
Produzione Industriale
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di Napoli Federico II



**Scuola Politecnica e
delle Scienze di Base**



Università degli Studi di Napoli Federico II

PHD PROGRAM IN

INDUSTRIAL PRODUCTS AND PROCESSES ENGINEERING

XXXVII CYCLE

Thesis for the Degree of Doctor of Philosophy

by

GENNARO LONGOBARDO

**Platforms Based On Micro- And Nano-shaped
Natural Materials For Lung Regenerative
Medicine And Biomedical Applications**



SCUOLA POLITECNICA E DELLE SCIENZE DI BASE

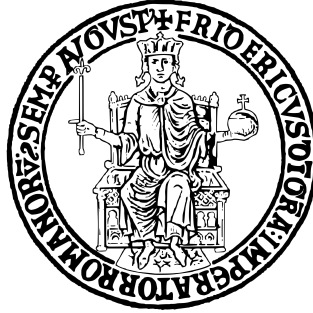
DIPARTIMENTO DI INGEGNERIA CHIMICA DEI MATERIALI E DELLA PRODUZIONE
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*Our greatest weakness lies in giving up.
The most certain way to succeed is always to try
just one more time.*

-Thomas Alva Edison



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Platforms based on micro- and nano-shaped natural materials for
lung regenerative medicine and biomedical applications

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*A Ivana,
per avermi donato delle passioni che colorassero la mia vita.*

*A Marina,
per avermi insegnato il valore della conoscenza e della dedizione.*

*A coloro che ho incontrato dentro e fuori da questo percorso,
e che mi sono stati d'aiuto nella mia crescita umana ancor prima che professionale.*



Sommario

È previsto che le malattie polmonari, come dichiarato dall'Organizzazione Mondiale della Sanità (OMS), entro il 2030, provocheranno un decesso su cinque a livello mondiale. Molte di queste patologie derivano da disfunzioni immunitarie e difetti nella riparazione del tessuto polmonare, in particolare nella regione alveolare, causando un anomalo rimodellamento dell'ECM e una progressiva perdita di funzionalità respiratoria. Le cellule alveolari di tipo II, fondamentali per la funzione polmonare, risultano particolarmente vulnerabili ai danni esterni, ma le attuali terapie disponibili sono soltanto di tipo palliativo.

I composti naturali, per la loro biomimesi, biocompatibilità e biodegradabilità, sono usati come substrati per l'ingegneria del polmone e come piattaforme bioingegneristiche. Polimeri come acido ialuronico, collagene e alginato, oltre a composti come acido folico e urea, sono stati ottimizzati in composizione e peso molecolare in questo dottorato. L'uso di miscele e modifiche chimiche, inclusa la coniugazione con altre molecole, è stato esplorato per migliorare le proprietà di questi materiali.

Quindi lo scopo di questa tesi di dottorato è quello di sviluppare piattaforme micro e nano-strutturate a base di polimeri e composti naturali sia a scopo rigenerativo nelle patologie e infezioni polmonari che per la costruzione di modelli tridimensionali polmonari. In particolare:

- La prima parte della tesi riguarda lo sviluppo di una piattaforma microstrutturata costituita da polimeri naturali e cellule staminali mesenchimali (MSCs) in una sospensione bifasica biomimetica. Lo scopo è quello di promuovere la rigenerazione e potenzialmente invertire il danno tissutale del polmone, ottimizzando il rilascio delle MSCs, aumentandone la ritenzione nei siti polmonari danneggiati e favorendone l'adesione mirata. In particolare, sono state ottenute microsfele di collagene senza l'uso di crosslinker chimici per il rilascio prolungato di MSCs e la loro differenziazione in ATII, e sono state sospese in una soluzione di acido ialuronico a basso peso molecolare iniettabile biomimetico.
- Successivamente, è stata affrontata la problematica delle infezioni polmonari, associate ad un accelerato deterioramento delle funzionalità polmonari a seguito dei danni nelle forme sia acute che croniche. Pertanto, si è proposto l'uso di acido folico per produrre *carbon dots* (CDs), una classe di nanoparticelle con precisa chimica superficiale, dotate di biocompatibilità verso i fibroblasti polmonari umani e capaci di superare il crescente problema della resistenza ai farmaci, mostrando attività antimicrobica. Specificatamente questi CDs hanno mostrato proprietà strutturali dipendenti dalle procedure di sintesi, le quali sono state poi correlate alla attività biologiche.
- In questo contesto è nata la necessità di ottimizzare e validare un nuovo costrutto biomimetico 3D del tessuto polmonare tramite tecniche di microfabbricazione, al fine di fornire un modello più efficiente e realistico, in grado di imitare la complessità delle vie aeree distali, con particolare attenzione alle interazioni tra le diverse cellule, quali fibroblasti e cellule epiteliali polmonari. Così è stato possibile ottenere un costrutto innovativo che può essere scalato comodamente capace di funzionare come una promettente piattaforma di studio biomimetica per la modellazione del tessuto polmonare sia sano che fibrotico a seguito di un insulto infiammatorio.

I risultati hanno mostrato nel complesso quindi l'estrema versatilità di questi materiali naturali, poichè capaci di essere adattati alla specifica applicazione di interesse, dalla medicina rigenerativa del tessuto polmonare alla fabbricazione di costrutti per l'ingegneria polmonare.

Abstract

By 2030, according to the World Health Organization (WHO), pulmonary diseases are expected to cause one in five deaths globally. Many of these conditions arise from immune dysfunctions and defects in lung tissue repair, particularly in the alveolar region, leading to abnormal extracellular matrix (ECM) remodeling and progressive respiratory function loss. Type II alveolar cells, essential for lung function, are particularly vulnerable to external damage, yet current therapies are only palliative.

Natural compounds, due to their biomimicry, biocompatibility, and biodegradability, are used as substrates for lung engineering and bioengineered platforms. Polymers like hyaluronic acid, collagen, and alginate, in addition to compounds like folic acid and urea, have been optimized in terms of composition and molecular weight throughout this doctorate work. The use of mixtures and chemical modifications, including conjugation with other molecules, has been explored to enhance these materials' properties.

Therefore, the goal of this PhD thesis is to develop micro- and nano-structured platforms based on polymers and natural compounds, both for regenerative purposes in pulmonary diseases and infections and for constructing three-dimensional lung models. Specifically:

- The first part of the thesis focuses on developing a microstructured platform made of natural polymers and mesenchymal stem cells (MSCs) in a biomimetic biphasic suspension. This platform aims to promote lung tissue regeneration and potentially reverse tissue damage by optimizing MSC release, enhancing retention at damaged lung sites, and encouraging targeted adhesion. Notably, collagen microspheres were created without using chemical crosslinkers for the sustained release of MSCs and their differentiation into ATII cells, suspended in an injectable, low-molecular-weight, ECM-like hyaluronic acid solution.
- Next, the challenge of lung infections, linked to accelerated functional decline from acute and chronic damage, was addressed. As a solution, folic acid was used to produce *carbon dots* (CDs), a class of nanoparticles with specific surface chemistry, biocompatibility toward human lung fibroblasts, and antimicrobial activity that counters the increasing issue of drug resistance. These CDs exhibited synthesis-dependent structural properties, which were then correlated with their biological activities.
- Within this context arose the need to optimize and validate a novel 3D biomimetic lung construct using microfabrication techniques to create a more efficient and realistic model. This model aims to replicate the complexity of the distal airways, focusing on interactions among different cell types, such as fibroblasts and pulmonary epithelial cells. This led to the development of an innovative construct that can be conveniently scaled, functioning as a promising biomimetic study platform for modeling both healthy and fibrotic lung tissue following inflammatory insult.

The results collectively demonstrate the remarkable versatility of these natural materials, as they can be tailored to specific applications, from regenerative lung tissue medicine to the fabrication of constructs for pulmonary engineering.

Contents

1	Introduction: state of art of lung tissue engineering	5
1.1	Lung tissue engineering	7
1.1.1	Lung tissue: ECM, function and histophysiology	7
1.1.2	The clinical problem and the limitations of conventional therapies in lung diseases	13
1.1.3	The clinical problem of pulmonary infections and the limitations of therapies due to multidrug resistance	17
1.2	Natural biomaterials for lung tissue engineering	19
1.2.1	Collagen (Col)	22
1.2.2	Hyaluronic acid (HA)	24
1.2.3	Heparin	25
1.2.4	Alginate (Alg)	26
1.3	Stem cells therapy for tissue engineering	28
1.3.1	Stem cells: properties and function	28
1.3.2	Stem cells for regenerative medicine	32
1.3.3	Stem cells for lung tissue engineering	33
1.4	Biomimetic approach to lung tissue engineering	35
1.4.1	The study of extracellular matrix	35
1.4.2	The use of lung 3D constructs versus 2D cell culture and <i>in vivo</i> models .	36
	Aim of the PhD thesis	40
2	Collagen-HA biphasic stem niche delivery systems for pulmonary differentiation	45

2.1	Introduction	47
2.2	Materials and methods	51
2.2.1	Materials	51
2.2.2	HA solution rheological characterization	51
2.2.3	Cell culture and mCOLLs preparation	52
2.2.4	mCOLLs diameter characterization and injectability by rheological characterization	54
2.2.5	mCOLLs viability and morphology	56
2.2.6	ATII expression study	57
2.2.7	Statistical analysis	57
2.3	Results and discussion	58
2.3.1	HA solution rheological characterization	58
2.3.2	MSCs stemness phenotype evaluation by flow citometry	60
2.3.3	mCOLLs diameter characterization	60
2.3.4	Rheological characterization	65
2.3.5	Cell delivery of MSCs from mCOLLs	67
2.4	Conclusions	69
3	Folic Acid-based carbon dots: synthesis optimization for enhanced antimicrobial activity against lung-related pathogens	70
3.1	Introduction	72
3.2	Materials and methods	75
3.2.1	Microwave-assisted synthesis, purification and yield	75
3.2.2	Zeta potential measurements	76
3.2.3	Fourier-Transformed Infrared (FT-IR)	77
3.2.4	Thermogravimetric analysis (TGA) of precursors and FA-CDs	77
3.2.5	UV-Vis absorbance	77
3.2.6	Biological properties	78
3.2.7	Antimicrobial activity	79
3.3	Results and discussion	80
3.3.1	Microwave assisted CDs synthesis from FA and urea	80

3.3.2	Zeta potential measurement	81
3.3.3	Fourier-Transformed Infrared (FT-IR)	87
3.3.4	Thermogravimetric and Differential Thermogravimetric analysis (TGA and DTG) of precursors and CDs	88
3.3.5	UV-vis absorbances	91
3.3.6	NHLFs cells viability assay and morphological analysis	92
3.3.7	Antimicrobial activity and oxidase-like activity by monitoring ROS generation	95
3.4	Conclusions and future perspectives	98
4	Design and optimization of a 3D biomimetic alveolar lung tissue construct	100
4.1	Introduction	102
4.2	Materials and methods	106
4.2.1	Materials	106
4.2.2	Cells culture	106
4.2.3	Alginate microbeads (mAlgs) fabrication and characterization	107
4.2.4	Collagen/Hyaluronic Acid hydrogel for 3D alveolar construct	110
4.2.5	mAlgs seeding density optimization in Alexa-Fluor-488 labelled Col/HA hydrogel and 3D alveolar construct characterization	110
4.2.6	Fibroblasts seeding density optimization in 3D alveolar constructs	111
4.2.7	Rheological characterizations	111
4.2.8	Coculture characterization in 3D alveolar construct	114
4.3	Results and discussion	117
4.3.1	mAlgs synthesis and characterization	117
4.3.2	mAlgs seeding density optimization	121
4.3.3	Effects of EDTA treatment: mechanical properties and NHLFs viability	123
4.3.4	NHLFs seeding density optimization and viability in time	124
4.3.5	A549 encapsulation in mAlgs	126
4.3.6	3D coculture system optimization	129
4.3.7	Inflammatory insult by PMA treatment: effect on alveolar walls formation	133

4.3.8	Inflammatory insult by PMA treatment: effect on cytokines expression, NHLFs activation and fibrotic response	137
4.3.9	Conclusions and future perspectives	144
Conclusions and future perspectives		146
Nomenclature		151
A Critical gel theory		152
A.0.1	Analysis for a physical thermosensitive gel	157
A.0.2	Analysis for a chemically crosslinked gel	159
B Possible folic acid and urea degradation and reaction path to form FA-CDs		161
C MATLAB code for 2d planar fit		165
D Thixotropic behavior of mAlgs suspension		167
E Optimization of epithelial cells encapsulation in mAlgs		173
Bibliography and sitography		178

Chapter 1

Introduction: state of art of lung tissue engineering

Contents

1.1 Lung tissue engineering	7
1.1.1 Lung tissue: ECM, function and histophysiology	7
1.1.2 The clinical problem and the limitations of conventional therapies in lung diseases	13
1.1.3 The clinical problem of pulmonary infections and the limitations of therapies due to multidrug resistance	17
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1.3.1 Stem cells: properties and function	28
1.3.2 Stem cells for regenerative medicine	32
1.3.3 Stem cells for lung tissue engineering	33
1.4 Biomimetic approach to lung tissue engineering	35

1.4.1	The study of extracellular matrix	35
1.4.2	The use of lung 3D constructs versus 2D cell culture and <i>in vivo</i> models	36

1.1 Lung tissue engineering

1.1.1 Lung tissue: ECM, function and histophysiology

Lungs are primary organs of respiration, located in the thoracic cavity and lie on either side of the mediastinum surrounded by the right and left pleural cavities, thus covered by a thin double-layered serous membrane called *pleura*. In particular, the right lung is normally a little larger than the left lung because the middle mediastinum, containing the heart, bulges more to the left than to the right [1].

The respiratory system -on the whole- has many functions, besides helping inhalation and exhalation -delivering oxygen to the cells in the whole body-, nonetheless fundamental [2]:

- It allows to talk and smell;
- It warms air in order to match body temperature and moisturizes it to the humidity level your body needs;
- Removes waste gases, including carbon dioxide, from the body during exhaling;
- Protects airways from harmful substances and irritants.

Respiratory system includes all the airways, lungs and blood vessels, as well as the muscles that power lungs. Then, it can be divided into two components:

- The *conducting portion*: it is the biggest one brings the air from outside to the site of the respiration. It can be schematized as series of pipes and chambers capable to filter and transport air from the outside to the inside. It is composed by smooth epithelial tissue.
- The *respiratory portion*: it helps in the exchange of gases and oxygenation of the blood. Then, its tissue needs to be more thin and porous for the molecules of gas to escape. This portion is connected to the other through the *trachea*.

As a matter of fact, trachea splits in the two *bronchi* and as far as histology is concerned they are very similar to trachea's respiratory epithelium. Furthermore, scattered throughout the

bronchi small cluster of neuroendocrine cells (called *Kulchitsky cells* [3]) can be found secreting *catecholamines*, hormones like adrenaline, serotonin, calcitonin and bombesin.

Eventually, bronchi splits into bronchioles¹ and thus histology changes, transitioning from respiratory epithelium (in particular a *ciliated pseudostratified columnar epithelium*) to a *ciliated simple columnar epithelium*, made up of a single layer. Getting deeper in pulmonary tree, arespiratory bronchioles are found, where *alveoli* are located.

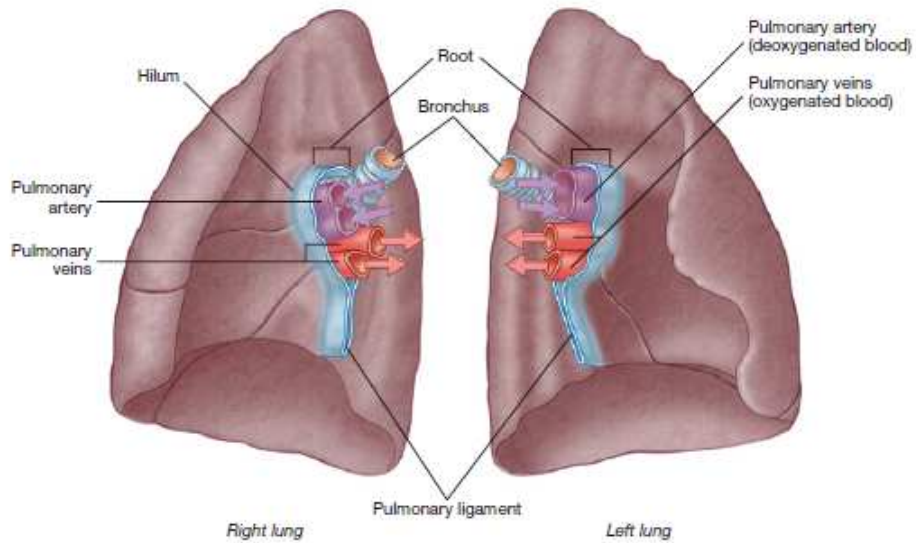


Figure 1.1: Lung anatomy, with particular emphasis on roots and hila of the lungs. *Image is adapted from Drake, R. L. e.(2016). Gray's basic anatomy. Philadelphia: Elsevier/Churchill Livingstone [1].*

It is fundamental now, in order to better understand the current advancements in the state of art of lung tissue engineering (LTE), to first focus on the role of such tissue, as well as its histology and specifically its ECM composition (see also **subsection 1.4.1**).

Lung ECM, it is quite unique, providing structural support for cells, and is critical in the regulation of developmental organogenesis, homeostasis and injury-repair responses and is severely affected by pulmonary pathologies (see **subsection 1.1.2**).

¹Anyting narrower than 1mm is classified as bronchiole, while bronchi have dimension larger than 1 mm.

Even though it share the general properties of being a dynamic network of fibrous proteins, glycoproteins, and proteoglycans, in the case of this specific tissue is typically restricted to two basic compartments [4]:

- *Basement membranes*: thin and specialized layers of glycoproteins composing ECM and can be found covering all epithelial and endothelial cell layers [5];
- *Interstitial spaces*: they form the parenchyma² of the lung by creating a loose and fibril-like meshwork that interconnects structural cell types within tissues, thereby maintaining the three-dimensional cohesiveness and biomechanical characteristics of the lung [7]. Here fibroblasts reside enmeshed, until they are required for wound repair.

Each on them shows a different composition. In their work *Burgstaller, G. et al.* reported the mice lung ECM's proteome³ and calculated the relative protein mass fraction of the indicated gene categories by normalizing mass spectroscopy intensities to the theoretical number of tryptic peptides intensity-based absolute quantification (iBAQ in **Table 1.1-1.2**).

Studies on human decellularized lungs shown that the extracellular lung parenchyma is not only composed of collagens, elastin and glycosaminoglycans (GAGs), as traditionally thought. Other proteins were identified, such as matrix Gla protein and nephronectin, that have not previously been associated with human idiopathic pulmonary fibrosis (IPF⁴) [10].

Nonetheless, it is notably assessed that composition and topography of lung ECM changes over the course of lung development, and can be highly heterogeneous, depending on location and lung's developmental stage, as reviewed by *Zhou, Y. et al.* [11]. As an example fetal mice lung tissues contain more total GAGs and proteoglycans (PGs), and higher expression of Col-I and Col-III in the pleura and the alveolar septae⁵, in comparison to adult tissues [12].

²Defined as "the essential and distinctive tissue of an organ or an abnormal growth as distinguished from its supportive framework" by *Merriam-Webster Dictionary* [6]

³Defined as "the complement of proteins expressed in a cell, tissue, or organism by a genome" by *Merriam-Webster Dictionary* [8]

⁴Defined as "chronic inflammatory progressive fibrosis of the pulmonary alveolar walls, with steadily progressive dyspnea, resulting in death from oxygen lack or right heart failure" [9]

⁵*Alveolar septum* separates neighboring alveoli.

Table 1.1: Collagen types and Proteoglycans ranked by their abundance in mice lung ECM [7]

Gene name	iBAQ ($\log_{10}$)	Protein name
Col6a1	9.36	Collagen alpha-1(VI) chain
Col6a3	9.31	Protein Col6a3
Col6a2	9.16	Collagen alpha-2(VI) chain
Col4a1	8.63	Collagen alpha-1(IV) chain
Col4a2	8.58	Collagen alpha-2(IV) chain
Col1a2	4.48	Collagen alpha-2(I) chain
Col1a1	8.18	Collagen alpha-1(I) chain
Col4a5	8.03	Col4a5 protein
Col12a1	7.98	Collagen alpha-1(XII) chain
Col18a1	7.96	Collagen alpha-1(XVIII) chain
Col14a1	7.94	Collagen alpha-1(XIV) chain
Col4a3	7.92	Collagen alpha-3(IV) chain
Col3a1	7.47	Collagen alpha-1(III) chain
Col5a1	7.46	Collagen alpha-1(V) chain
Col15a1	7.38	Collagen alpha-1(XV) chain
Col4a4	7.37	Collagen alpha-4(IV) chain
Col16a1	7.23	Collagen alpha-1(XVI) chain
Col4a6	7.16	Protein Col4a6
Col7a1	7.02	Collagen alpha-1(VII) chain
Col8a1	6.97	Collagen alpha-1(VIII) chain
Col6a6	6.93	Collagen alpha-6(VI) chain
Col5a2	6.60	Collagen alpha-2(V) chain
Col6a4	6.39	Collagen alpha-4(VI) chain
Col11a1	6.31	Collagen alpha-1(XI) chain
Col14a1	6.16	Collagen alpha-1(XIV) chain
Col10a1	6.07	Collagen alpha-1(X) chain
Col6a5	6.06	Collagen alpha-5(VI) chain
Col5a3	5.45	Collagen type V alpha 3 chain
Col6a3	5.40	Protein Col 6a3
Col28a1	5.20	Collagen alpha-1(XXVIII) chain
Col2a1	4.48	Collagen alpha-1(II) chain

Gene name	iBAQ ($\log_{10}$)	Protein name
Bgn	9.47	Biglycan
Hspg2	9.41	Perlecan
Lum	9.29	Lumican
Prep	9.01	Prolargin
Ogn	8.99	Mimecan
Prg2	8.98	Bone marrow proteoglycan
Dcn	8.98	Decorin
Aspn	8.53	Asporin
Podn	7.51	Podocan
Prg3	7.51	Proteoglycan 3
Chad	7.10	Chondroadherin
Fmod	6.75	Fibromodulin
Vcan	6.32	Versican
Hapln1	6.25	Hyaluronan and proteoglycan link protein 1
Srgn	6.03	Serglycin
Omd	5.89	Osteomodulin
Acan	4.93	Aggrecan core protein
Prg4	4.3	Proteoglycan 4

Table 1.2: Principal glycoproteins ranked by their abundance in mice lung ECM [7]

Gene name	iBAQ ($\log_{10}$)	Protein name	Gene name	iBAQ ($\log_{10}$)	Protein name
Mfap4	9.59	Microfibril-associated glycoprotein 4	Wisp2	7.78	WNT1-inducible signaling pathway protein 2
Lamc1	9.53	Laminin subunit gamma-1	Thxb	7.78	Protein Thxb
Lamb2	9.46	Laminin subunit beta-2	Sparcl1	7.77	SPARC-like protein 1
Nid1	9.44	Nidogen-1	Tinag	7.71	Protein Tinag
Postn	9.44	Perlestin	Lrg1	7.68	Leucine-rich HEV glycoprotein (Precursor)
Fbln5	9.39	Fibulin-5	Ntn4	7.67	Netrin-4
Lamb3	9.27	Laminin subunit beta-3	Mmrn2	7.62	Multimerin-2
Lama5	9.21	Laminin subunit alpha-5	Ecm1	7.6	Extracellular matrix protein 1
Lama2	9.18	Laminin subunit gamma-2	Pxdn	7.6	Peroxidasin homolog
Lama3	9.08	Laminin subunit alpha-3	Ntn1	7.58	Netrin-1
Fga	9.07	Fibrinogen, alpha polypeptide	Papln	7.57	Papilin
Dpt	8.94	Dermatopontin	Abi3bp	7.52	Protein Abi3bp
Emilin1	8.9	EMILIN-1	Fbln1	7.45	Fibulin-1
Fnl1	8.85	Fibronectin	Pcolce	7.44	Procollagen C-endopeptidase enhancer 1
Fgb	8.84	Fibrinogen beta chain	Fbln1	7.43	Fibulin-1
Nid2	8.83	Nidogen-2	Ltbp2	7.4	Latent-transforming growth factor beta-binding protein 2
Npnt	8.79	Nephronectin	Vval	7.37	von Willebrand factor A domain-containing protein 1
Fgg	8.78	Fibrinogen gamma chain	Fbln2	7.29	Fibulin-2
Lama4	8.71	Laminin subunit alpha-4	Creld2	7.29	Cysteine-rich with EGF-like domain protein 2
Adipoq	8.57	Adiponectin	Sned1	7.26	Sushi, nidogen and EGF-like domain-containing protein 1
Tinagl1	8.55	Tubulointerstitial nephritis antigen-like	Matn4	7.26	Matrilin-4
Agrn	8.52	Agrin	Thbs3	7.25	Thrombospondin-3
Mgp	8.49	Matrix Gla protein	Ltbp4	7.21	Latent-transforming growth factor beta-binding protein 4
Vwa5a	8.48	von Willebrand factor A domain-containing protein 5A	Tnc	7.2	Tenascin
Vtn	8.45	Vitronectin	Igfbp5	7.17	Insulin-like growth factor-binding protein 5
Eln	8.44	Elastin	Slit3	7.16	Slit homolog 3 protein
Tgfb1	8.4	Transforming growth factor-beta-induced protein ig-h3	Igfbp6	7.15	Insulin-like growth factor-binding protein 6
Efemp1	8.39	EGF-containing fibulin-like extracellular matrix protein 1	Pcolce2	7.1	Procollagen C-endopeptidase enhancer 2
Igfbp7	8.39	Insulin-like growth factor-binding protein 7	Aebp1	7.08	Adipocyte enhancer-binding protein 1
Sparc	8.3	SPARC	Svep1	7.01	Sushi, von Willebrand factor type A, EGF and pentraxin
Vwf	8.29	von Willebrand factor	Igfals	6.98	Insulin-like growth factor-binding protein complex
Mfge8	8.25	Lactadherin	Postn	6.94	Periostin
Mfap2	8.24	Microfibrillar-associated protein 2	Efemp2	9.88	EGF-containing fibulin-like extracellular matrix protein 2
Lama2	8.22	Laminin subunit alpha-2	Colq	6.88	Acetylcholinesterase collagenic tail peptide
Spon1	8.15	Spondin-1	Hmnc1	6.86	Protein Hmnc1
Fbn1	8.06	Fibrillin-1	Srpax2	6.85	Sushi repeat-containing protein SRPX2
Ltbp1	7.88	Latent-transforming growth factor beta-binding protein 1	Mmrn1	6.83	Multimerin-1
Emid1	7.86	EMI domain-containing protein 1	Egflam	6.72	Pikachurin
Thbs1	7.81	Thrombospondin 1	Bimpr	6.72	BMP-binding endothelial regulator protein
Mfap5	7.8	Microfibrillar-associated protein 5	Lama1	6.71	Laminin subunit alpha-1

It is known for a long time and nowadays ascertain that fibrillar proteins, such as collagens and fibronectins, account for the tensile strength of the lung, while molecules such as elastin (and collagen) account for its elastic recoil, a fundamental aspect for lung physiology [13, 14]. Thus, any modifications in the levels and ratios of these proteins -among others- thus alter relative contributions of each molecule to ECM stiffness.

Focusing more on elastin, it is a averagely not high molecular weight fibrous protein (about 72 kDa), which constitutes the other fiber of the ECM fabrics. Being less complex than collagen, in terms of structuring, elastin does not contain iridoxypoline, but contains hydrophobic amino acids (alanine, valine, leucine, glycine), capable of interacting with other amino acids hydrophobic to form lattices (**Figure 1.2**).

Therefore, it is characterized by an extensive crosslinking (desmosine, iso-desmosine, lysine-norleucine). It has a relaxed conformation of random coil and assumes an elastic behavior whenever mechanically stressed [15]. Furthermore, it has been shown that elastin is the most important factor in determining the recoil for small lung volumes, as the volume increases the collagen begins to take over [16, 17].

Hyaluronic acid (HA) in lung parenchyma is a key component of the ECM and plays a main role in water regulation in the interstitium [18]. HA modulates neutrophil elastase secretion, stimulates or represses immunologic reactions, suppresses bronchial responsiveness, and may play a role in alveolar surface structure stabilizing the surfactant proteins [19].

Lately, in respiratory medicine, HA has been successfully evaluated in the treatment of a number of lung diseases in experimental animal models and humans, due to its ability to relieve symptoms.

It has been determined that the exogenous HA administered by aerosol or tracheal instillation acts against inflammation, protecting the airways against the hyper-reactivity and remodelling of the bronchial and lung parenchyma [20].

For example, in patients affected by chronic lung disorders, repeated administrations of inhaled HA, daily, for 8 weeks, induce significant increase in bronchial patency as well as progressive lung deflation with decrease of residual volume [21].

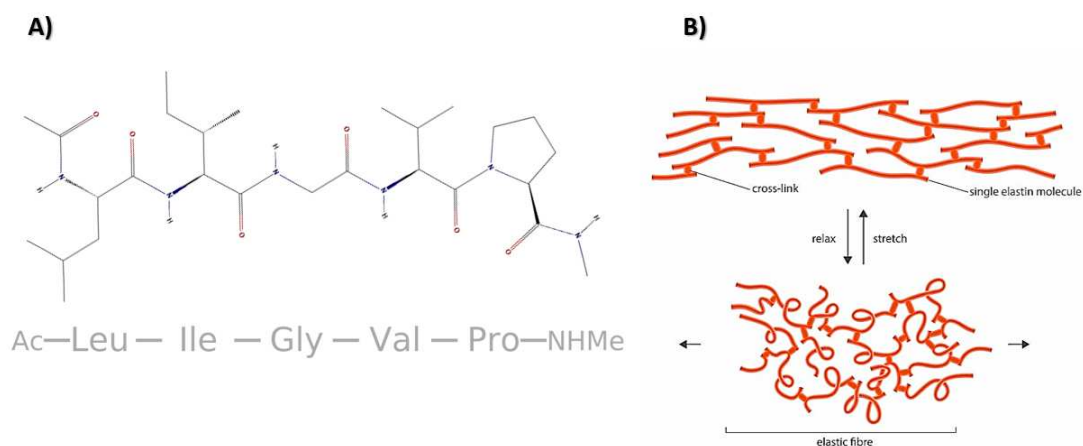


Figure 1.2: A) Elastin molecular structure and biologic description in terms of component amino acids. B) Elastin coil and crosslinking. Images adapted from National Center for Biotechnology Information. "PubChem Compound Summary, <https://pubchem.ncbi.nlm.nih.gov/compound/Elastin>. [22].

1.1.2 The clinical problem and the limitations of conventional therapies in lung diseases

Chronic pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), asthma and Bronchopulmonary dysplasia (BPD) of the preterm infant, are chronic inflammatory diseases characterized by airway obstruction up to an alteration in the function of the alveoli in the case of COPD and from a lack of alveolar development of the premature baby, with the presence of dysmorphic alveoli and persistent inflammatory situation and subsequent fibrosis of the lung tissue, in the case of BPD [23].

In particular, one of the most troublesome pathology is COPD, mainly caused by smoke exposure: a key factor of COPD is airway remodelling, which refers to the serious alterations of the ECM affecting airway wall thickness, resistance and elasticity [5].

In these pathologies, the absence of resolution of the pulmonary lesions leads to an aberrant repair of the tissue, causing a decrease both at the numerical and functional level of the fundamental unit of the lungs, or rather of the alveoli, and more particularly of the alveolar cells of type II (ATII) which are directly involved in the production of pulmonary surfactant.

The surfactant has to maintain the correct pulmonary biophysical behavior thanks to

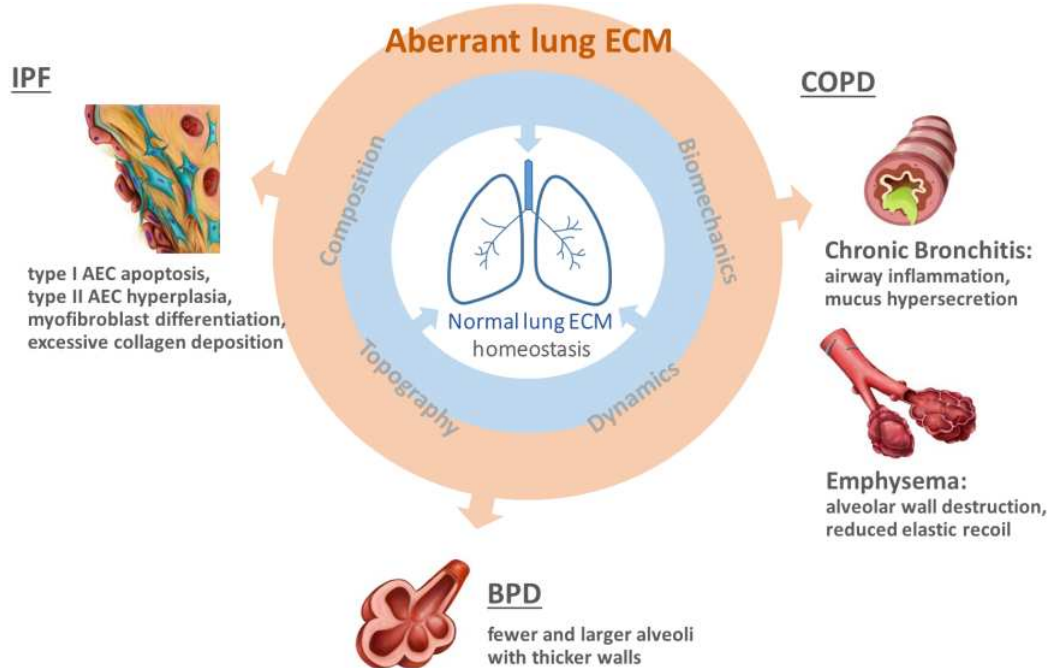


Figure 1.3: Some possible aberrant alterations of the properties of lung ECM in composition, biomechanics, dynamics and topography. Here IPF= idiopathic pulmonary fibrosis; COPD= chronic obstructive pulmonary disease; BPD= bronchopulmonary dysplasia.

the decrease in surface tension within the pulmonary alveoli, thus decreases the risk of collapse during exhalation, handles immunomodulatory action and regulate the correct alveolar size.

Below, an overview of the lung and sites of disease formation is depicted (**Figure 1.4-a**), as well as all main lung pathologies outcomes are summarized [24]. In an healthy lung, interstitial ECM is maintained by the activity of resident fibroblasts and represents a loose meshwork of collagens, elastin and fibronectin anchored to the basal membrane of the epithelial cell layer, as described before (**Figure 1.4-b**).

On the other hand, in IPF repetitive lung injury and subsequent repair mechanisms lead to an ongoing destruction of elastic parenchymal lung tissue. Fibroblasts transdifferentiate to highly contractile myofibroblasts, which increases ECM rigidity by enzymatic covalent crosslinking of collagen and elastin. All these changes in composition contribute to a progressive loss in gas exchange, with subsequent deteriorating lung function, breathlessness and finally respiratory failure leading to death (**Figure 1.3**, **Figure 1.4-c**).

The hallmarks of pathological changes within the ECM in COPD are, instead, the extensive destruction of elastic fibres by ECM-degrading enzymes released by inflammatory cells,

along with elevated levels of HA and tenascin-C, and decreased deposition of decorin. This leads to airflow obstruction and hyperinflation, due to the destruction of the epithelial cell layer and thus the formation of air space enlargements or *emphysema* (**Figure 1.4-d, Figure 1.5**).

In Pulmonary Arterial Hypertension (PAH), remodelling of the ECM within the arterial wall bring to an increased workload in the right heart ventricle. In fact, there is an increase in elastin and Col fibres, fibronectin, and tenascin-C, and hyperplasia of smooth muscle cells, as the whole three-layered architecture of the arterial wall undergoes remodelling, thus leading to intimal, medial and adventitial thickening. (**Figure 1.4-e**).

In asthma takes place a chronic inflammation of submucosal regions of the small airways of the lung, with associated goblet cell hyperplasia, augmented mucus secretion, smooth muscle hypertrophy and bronchoconstriction. The characteristic ECM changes take place beneath the bronchial epithelium and thickened basal membrane. In addition, smooth muscle cells deposit Coll, fibronectin, HA and decorin are increased (**Figure 1.4-f**).

Eventually, in cancer, tumours at primary and metastatic sides are surrounded by an extensive stiff stroma that contains highly crosslinked Col-IV, and high levels of fibronectin, tenascin-C and hyaluronan. Some studies found out how the patients survival time was shortened, when the small cell lung cancers bind to the ECM, which protects the cancer cells from chemotherapy-induced apoptosis [25] (**Figure 1.4-g**).

Currently there are no effective treatments following alveolar damage for these pulmonary pathologies, but replacement technologies are put in place in the forms of cardiopulmonary bypass and extracorporeal membrane oxygenation.

Still, such solutions are restricted only to intensive care unit. In their work, *Camboni et al.* managed to test on two patients for the first time a paracorporeal artificial lung (PAL) in parallel to the pulmonary circulation, posed in connection to the pulmonary artery and to the left atrium. Out of two, only one survived and his pulmonary hypertension was supported 62 days, preceding a lung transplantation [26].

New frontiers for lung regenerative medicine are found, in last times, among cellular based LTE technologies, trying to mimic the incredible complexity of one among the most

remarkable of human tissues, which accommodates a 0.5 μm thick respiratory membrane for O_2 and CO_2 exchange.

Thus, lung tissue biomimetic regeneration through bio-engineered scaffolds is a quite challenging issue, having to face the extremely complex three dimensional anatomical structure of the lung. As an example, differently from other tissues (e.g. cartilage tissue), both vascular and airway structures are required, in order to prevent tissue necrosis as well as to grant a functional gas exchange [27]. In particular, there is a new line based on stem cells (see **subsection 1.3.3**), thanks to their multilinear differentiation potential, for their immunomodulatory properties and for the ease of isolation.

In fact, stem cells, thanks to their ability to differentiate according to certain stimuli of the niche/tissue environment in ATII, would allow the repopulation of the compromised areas. Moreover, through paracrine mechanisms they would favor the release of pro-angiogenic and anti-apoptotic factors, with beneficial effects on lung chronic lesions [16, 17].

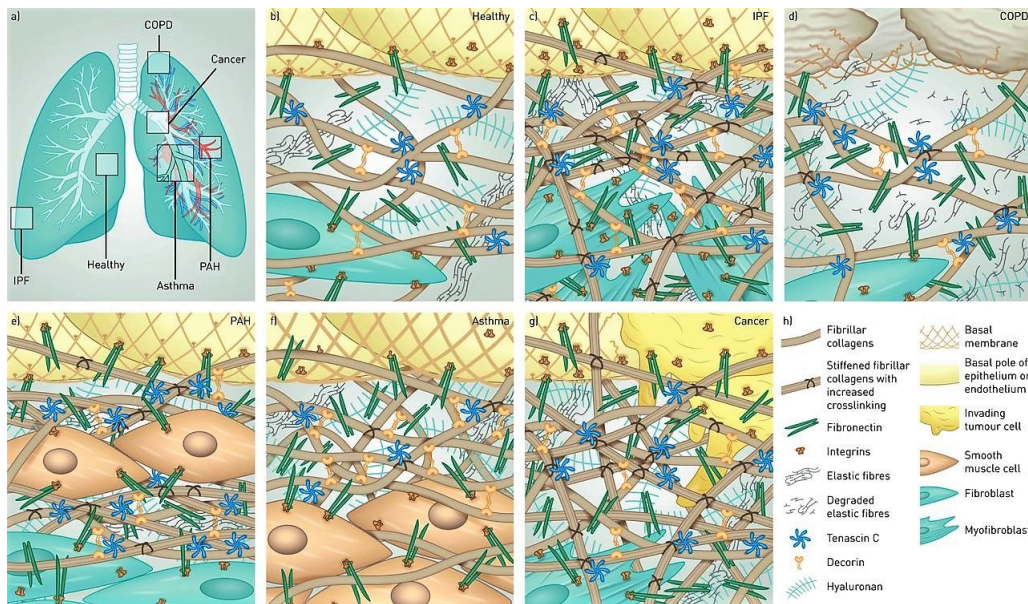


Figure 1.4: Pathological changes within the interstitial extracellular matrix (ECM) in the diseased lung (*Image adapted from Burystaller, Gerald, et al. "The instructive extracellular matrix of the lung: basic composition and alterations in chronic lung disease." European Respiratory Journal 50.1 (2017)[24].*

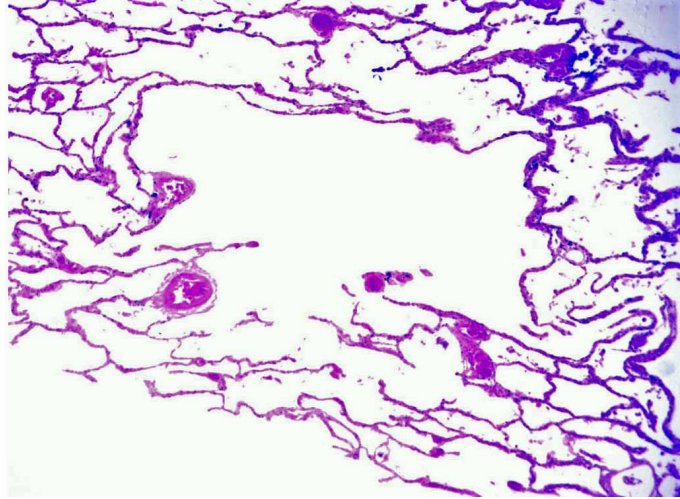


Figure 1.5: In the emphysematous lung, air spaces become enlarged due to increased compliance and destruction of the alveolar walls. Proteolysis of elastic fibers, within the alveolar walls increases the compliance of the walls and then their destruction. (*Image adapted from <http://medcell.med.yale.edu/>*).

1.1.3 The clinical problem of pulmonary infections and the limitations of therapies due to multidrug resistance

The pulmonary diseases caused by bacteria infections are also a major clinical challenge: they often result in acute respiratory complications, which can escalate to life-threatening situations such as acute respiratory distress syndrome (ARDS). While chronic inflammatory lung diseases typically involve long-term airway inflammation and structural modifications, bacterial infections usually result in immediate immune responses that cause rapid onset symptoms like coughing, fever, and dyspnea.

Typically, the absence of prompt treatment for bacterial pneumonia and bronchitis can result in severe complications such as lung abscesses and systemic infections. While chronic diseases like COPD involve progressive deterioration over time, bacterial infections necessitate timely intervention to prevent acute exacerbations and preserve lung function, underscoring the need for effective diagnostic and therapeutic strategies tailored to these distinct yet interrelated pulmonary issues.

Furthermore, when dealing with bacterial infections, a fairly new issue has been emerging in recent times: multidrug resistance (MDR). Such issue poses a critical challenge in the management of pulmonary infections, significantly complicating treatment options for conditions such as pneumonia and bronchiectasis.

MDR bacteria, have typically developed the ability to withstand the effects of multiple antibiotics in a very different set of ways (from the production of inactivating enzymes, to the transferring of resistance genes, biofilm formation, etc.). Such rapid development of antibiotic resistance in microorganisms is usually related to the overconsumption of antibiotics, their broad utilization in agriculture and also the non-availability of novel antibiotics [28].

This issue contributes to prolonged hospital stays, increased healthcare costs, and higher morbidity and mortality rates among affected patients. This resistance often arises from the overuse and misuse of antibiotics, leading to a cycle where standard treatments become ineffective. As a result, healthcare providers face difficulties in selecting appropriate antimicrobial therapies, prompting the need for innovative strategies to combat these resilient pathogens.

In case of pulmonary infections several bacterial strains in peripheral lung and upper airways are found and they can also show a MDR effect. In a study by *Pragman et al.* microbiota samples were obtained from nine subjects with mild or moderate COPD by swabbing lung tissue and upper airway sites during lung lobectomy and they found different bacterial strains at different sections of the upper and lower airways, such as *Streptococcus*, *Rothia*, *Veillonella*, etc [29].

Besides, another study by *Miravittles et al.* discussed a secondary role of bacteria that do not have a direct effect in terms of chronic inflammation, but that may bring acute bacterial exacerbations of a preexisting COPD pathologies. The strain they identified with such behavior were *H. influenzae* , *P. aeruginosa* and *Moraxella catarrhalis* [30].

In order to fight such resistance, nano-sized delivery systems are increasingly being evaluated as viable options for improving therapeutic efficacy. They can actually limit drug degradation once encapsulated, as well as increasing the accumulation at infection site, while also reducing the counter-effect of toxicity [28, 31].

As an example, loaded nanoparticles have the capacity to safely and effectively perform a wide variety of treatments. Actually, they can act as inherent therapies (e.g. Au NPs, Ag NPs, etc.), but can also work as nano-carriers for antimicrobial agents -like in the case of liposomes, polymeric NPs, and dendrimers [32].

Among the most effective and promising nano-sized substrates with antimicrobial properties, carbon dots (CDs) have been lately acquired interest [33]. The interest relies not only on their ease of synthesis and lower costs, but also on some specific advantages CDs shows when compared to nanoparticles.

Firstly, CDs can generate reactive oxygen species (ROS) that disrupt bacterial cell membranes, making them effective against resistant strains. Therefore, they show diverse modes of action with respect to conventional antibiotics and reduce the likelihood of bacteria developing resistance [34].

Additionally, CDs can be engineered for better bioavailability and targeted delivery, enhancing treatment efficacy while minimizing side effects. As a matter of fact, their chemical structure lends itself well to chemical modification. Moreover, carbon dots are generally biocompatible and usually obtained from natural compounds, potentially providing a safer profile compared to some nanoparticles, which might pose toxicity risks [35].

1.2 Natural biomaterials for lung tissue engineering

Impare tissue engineering is that branch of bio-engineering aiming to restore, replace or regenerate different types of tissues or organs. This is a unique and multidisciplinary branch, which borrows methods and perspectives from medicine, cell and molecular biology, materials science and engineering in order to understand the relationships between structure and function within the physiological or pathological tissues of mammals [36].

It also studies methodologies for the development of biological substitutes or substrates that can restore or even optimize the biological functions of these tissues.

More in detail, *soft tissue engineering* (sometimes shortened to STE) is a sub-field of tissue engineering, dealing with all tissues except bone. As a matter of fact, it focuses more on skin, musculoskeletal (i.e. cartilage, ligaments/tendons, muscle, connective tissues), cardiovascular, neural (i.e. brain, nerves), gastrointestinal, liver, ocular tissues and many others. Between those tissues, LTE is the sub-branch focusing on pulmonary tissue restoration.

In general, tissue engineering strategies require interaction between cells and biomaterial substrates or scaffolds. The choice of the specific substrate must be such as to allow the cells to behave as required, in order to be able to generate functional tissues or organs of the desired size. To be effective, a biomaterial scaffold must have the following characteristics:

- Possess interconnected pores of such dimensions as to facilitate the transport of substances, as well as the integration and vascularization -whenever needed- of the tissue;
- Be composed of materials such as to be able to control biodegradability or bioabsorbability, in such a way as to favour an effective replacement by the fabric;
- Be endowed by an appropriate surface chemistry, in order to favor cell adhesion, as well as their differentiation and proliferation. Tissue–biomaterial mechanical mismatch can result in mechanical failure if the biomaterial is too weak, induce inflammation and tissue damage if biomaterial stiffness is considerably higher than the one of the native tissue [37];
- Keep the mechanical structure of the organs, allowing not only the insertion, but also the use of the fabric in question with the least possible impediments;
- Avoid producing adverse responses from the body in the form, provoking at most minimal degree of toxicity, or inflammation;
- Being able to be manufactured in the widest possible variety of shapes, as well as dimensions, in order to favor a correct insertion and functioning.

Biomaterials used for STE show an incredibly vast range of properties, especially mechanical properties, as they change remarkably in native tissues, for example, elastic modulus can change considerably, going from brain tissue to cartilage tissue (**Fig.1.6**).

Such biomaterials are used to produce engineered tissues by scaffolding in two different approaches: scaffolds are used or as cell supporting system for seeding cells *in vitro* -and then stimulate matrix the production- or as a drug delivery device, by loading the scaffolds with grow factors (cells are recruited around the scaffold itself) [38].

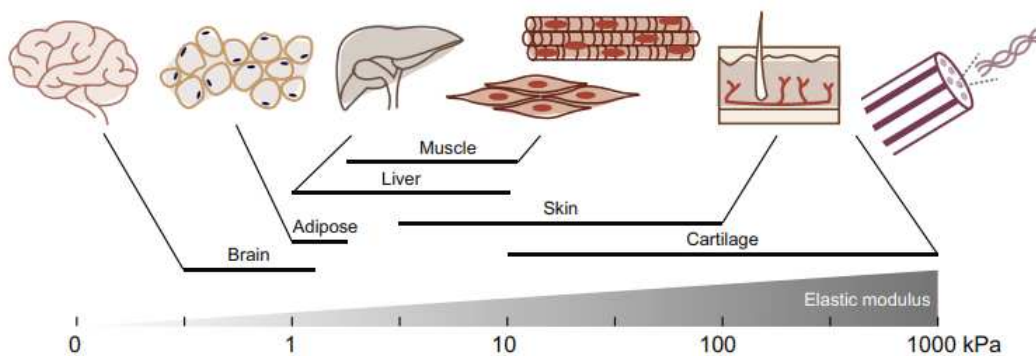


Figure 1.6: Range of elastic modulus of different types of soft tissues [39]

Synthetic polymers have been widely used in soft tissue engineering in the face of their high customization and their controlled and reproducible compositions. Application of synthetic polymers ensures high flexibility in the modulation of matrix architecture, its physicochemical properties, and their delivery kinetics for specific bioactive factors. That is the reason why many synthetic polymers have been screened for their suitability as injectable hydrogels for soft tissue engineering, especially cartilage tissue.

Thus, since most synthetic polymers are hydrophobic, some limitations in terms of cell attachment and differentiation may occur due to an insufficient number of interaction sites [40]. Therefore, blends of hydrophobic and hydrophilic polymers are sometimes used to enhance hydrophilicity and promote cell attachment [41].

In particular, in this study we are going to give more attention to a specific soft tissue: the lung tissue. Besides, it will be better analyzed what materials have been studied in literature for LTE, though focusing more on natural polymers.

As a matter of fact, differently from synthetic ones, natural polymers possess an inborn biocompatibility, biodegradability, and resemblance to the ECM and so to the native tissue, as well as enhanced cell adhesion, proliferation and differentiation properties.

Such properties have played an important role in decreeing their use in STE, as they embody bioactive components within itself, improving cell recognition, adhesion, proliferation, and biological repair. On the other hand, natural biomaterials show some quite disabling drawbacks, especially some poor mechanical properties, as well as a fast and unpredictable degradation under biological conditions.

Nevertheless, it is possible to work around such inconvenience, by chemical modification with addition of specific macromolecules or by the creation of interpenetrating networks, consisting of different polymers, whose chains interpenetrate one another in a dense 3D network [42].

To this reason it was made a large use of natural polymers in this PhD thesis, such as Collagen, hyaluronic acid, etc. For this reason it follows a list of the used natural polymers, which result to be also some commonly polymers in LTE, together with a summary table (**Table 1.3**).

1.2.1 Collagen (Col)

Collagen is a structural protein and it represents about 25% of all proteins in the human body. It is one of main constituents of soft tissues, supporting skin and internal organs; however, it occurs in hard tissues in different forms [43]. There are at least 19 distinct collagen types that have been delineated, sharing a common typical helical structure and distinct amino acid arrangement [44]. Collagen characteristics are due to amino acids in its structure (**Figure 1.7, A**). As a matter of fact collagen is identified by following structures:

- Primary structure: a single chain composed of 1400 or above amino acids and is characterized by a repetition of four amino acids, of which the first three are always the triplet Glycine (Gly), Proline (Pro) and Hydroxyproline (Hyp), while the fourth changes;
- Secondary structure: the α -*helix* is formed;
- Tertiary structure: a left-handed helix is formed;
- Quaternary structure: is the right-handed superhelix, or *tropocollagen*. It is made up three helices as sub-units and its development is axial, to form microfibrils, and then fibers, that can reach very large dimensions. The last two amino acids in primary structure favours the typical spiral winding of the chains, to form such right-handed helical structure, while glycine is small enough to place itself perfectly inside the triple helix (**Figure 1.7**).

Then, tropocollagen molecules associate with each other through non-binding interactions to form staggered parallel rows, called *submicroscopic microfibrils* (20-100 nm thick).

Eventually, these re-associate with each other along one direction to form *microscopic fibrils* (0.2-0.3 μm thick).

Collagen is probably the most commonly used natural polymer for LTE scaffold fabrication since it can be extracted from a wide range of sources. In addition, it shows an excellent biological compatibility, good biodegradability, low immunogenicity, strong cell adhesive ability, and an appropriate hydrophilicity. However, it is unstable: due to its rapid degradation rate, the scaffold is unable to maintain structural integrity, thus threatening its mechanical properties in time.

Focusing on culture of stem cells in Col-I (the main component in connective tissues) hydrogels for cartilage tissue engineering, it was found out that MSCs could be seeded in a Col-I matrix acting as a stem niche for their differentiation into pulmonary lineage, in particular Surfactant protein C- secreting ATII cells [45].

To enhance hydrogel stability and being able to tune its stiffness *Lu Z. et al.* loaded a collagen hydrogel with carbon dot nanoparticles: the composite hydrogel showed actually 21-fold higher compression modulus and a 39.3% lower degradation rate than the pure collagen hydrogel [46].

In their work *Yuan et al.*, instead, used Col-I and Col-II to create an injectable hydrogel where the compressive modulus of the hydrogel could be tuned by a modulation in the content of Col-I. [47].

Instead, studying the formation of lung histotypic structures in the tissue-engineering scaffolds, different works show the efficacy of Col as a lung tissue construct main component. In particular, *P. Chen et al.* designed a Col-I/GAG scaffold, by seeding it with fetal rat lung cells; eventually, dissociated lung cells could generate lung tissue physiological structures in the synthetic scaffold [48].

Same way, interesting and fundamental for the aims of this PhD thesis is the specific use of methacrylated Col-I as main component of a 3D biomimetic hydrogel capable of supporting lung cells seeding and proliferation in time, with mechanical properties very close to those belonging to the native tissue, as reported by *Nairon et al.* [49].

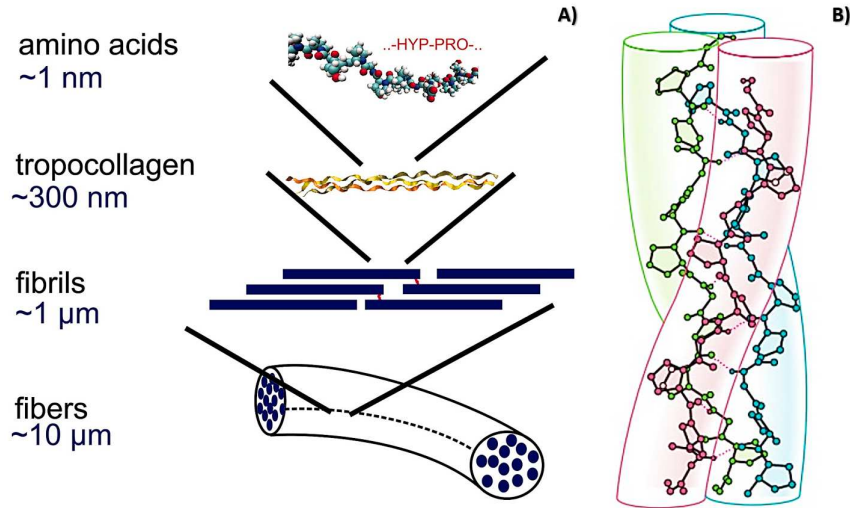


Figure 1.7: A) Schematic view of some of the hierarchical features of collagen; B) Tropocollagen triple helix magnification of Poly-(Gly-Pro-Hyp) composed of three left-handed helical chains; the three strands are held together by hydrogen bonding. (Adapted from Buehler, Markus J. "Nature designs tough collagen: explaining the nanostructure of collagen fibrils." *Proceedings of the National Academy of Sciences* 103.33 (2006) [50]).

1.2.2 Hyaluronic acid (HA)

Hyaluronic acid (or hyaluronan) is defined as a non-sulfated, linear polysaccharide consisting of alternate N-acetyl-d-glucosamine and d-glucuronic acid linked by $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycoside bonds (**Figure 1.8**) and belongs to the family of GAGs, that are generic polysaccharides [51]. In particular, HA is ubiquitous in soft connective tissue of all vertebrates.

Among its best known properties, HA has the ability to retain huge amounts of water - and therefore has a huge amount of bound water- which makes it the most adapted to regulate flows and to deal with transport processes, also thanks to its high solubility. Actually, thanks to its properties HA is widely used also in wound dressing applications under different morphologies: from moisture to films and hydrogels, from fibres to non-woven fabrics and foams.

Along its backbone HA chain shows a large number of negative charges, so when compressed it will tend to remove these charges for electrostatic repulsion. This results in a high viscosity of HA solutions. Indeed, tissues rich in HA (is present in all connective tissues, e.g. 10-20% of GAGs in the skin and aorta, 1% of GAGs in cartilage) possess very high viscosities.

In literature, it was also shown an optimal bacteriostatic effect of HA against *S. epidermidis* and *P. aeruginosa*, but in general against different Gram-positive and Gram-negative bacteria[52, 53]. Hence, it is theorized that the HA molecules also create an interconnected

structure of strands that can function as a barrier halting the migration of bacteria [54].

From the point of view of regenerative effect, HA-based hydrogel wound dressings are notable for their capacity to be an appropriate option for all four phases of wound healing, in addition to being non-irritating and non-reactive with biological tissue, while also allowing for the passage of metabolites [55].

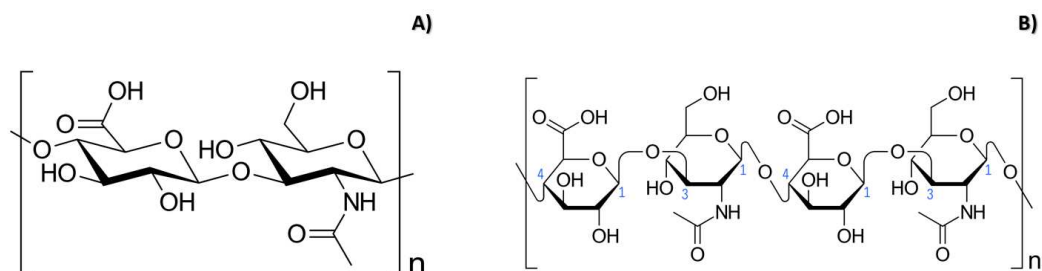


Figure 1.8: **A)** Skeletal formula of hyaluronan; **B)** Haworth projection of hyaluronan: this way $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycoside bonds are shown.

1.2.3 Heparin

Heparin is a highly sulfated, linear polysaccharide composed of repeating disaccharide units of 1,4-linked uronic acid and glucosamine residues, leaving it with the presence of reactive groups such as carboxyl and sulfate: this causes heparin to be easily chemically modifiable (**Figure 1.9**). Heparin is known mostly as an anticoagulant used as a treatment of superficial thrombophlebitis. Actually, heparin has also been studied in combination to various biomaterials to decrease the thrombogenicity of the materials in contact with blood. In addition to its anticoagulative properties, heparin scaffolds show antiangiogenic, anticancer and anti-inflammatory effects.

Its incorporation into lung tissue scaffolds can enhance biocompatibility and mimic native ECM environments, promoting cell adhesion, proliferation, and differentiation. In particular, heparin's interactions with growth factors [56] such as fibroblast growth factors (FGFs) can in theory support alveolar cell regeneration, which is crucial in repairing lung damage and modeling alveolar structures. As an example, a complete epithelial cells monolayer was generated on albumin/heparin-coated PMP gas exchange membranes, that were able to provided a bio-functional surface to be used in gas exchange devices which were in contact with blood [57].

Moreover, heparin-functionalized biomaterials have shown promise in creating microenvironments that resemble *in vivo* lung tissue applications for multiple purposes [58].

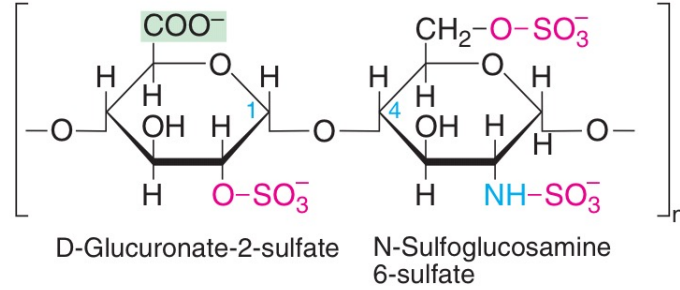


Figure 1.9: Structure of heparin; (1→4)-links are shown (Adapted from Satyanarayana and Chakrapani "Biochemistry, 4e", Elsevier (2013)) [59]

1.2.4 Alginate (Alg)

Alginate comes from brown algae or bacterial biofilms and is a linear polysaccharide: its monomers are β -D-mannuronic acid (M) and α -L-glucuronic acid (G). Each of the monomers confers different properties to this biomaterial: the monomer G will tend to give a greater crystallinity, being intrinsically more packaged; vice versa the monomer M will tend to increase the amorphous fraction, given its more linear structure.

It is a widely used biomaterial, thanks to its biocompatibility, biodegradability, non-antigenicity, chelating ability and mucoadhesive, as well as to its high hydrophilicity and water-solubility. On the other hand, alginate suffers from low mechanical properties and very poor binding affinity to serum proteins, which restricts the adhesion of certain mammalian cell types.

An interesting aspect about G monomers is the presence of COO^- carboxyl groups, each with a negative charge, capable of interacting with divalent cations, in particular calcium, zinc and strontium ions, thus forming the so called *egg-box* (**Figure 1.10**). This interaction represents the formation of crosslinks in alginate-based hydrogels: the interaction that is established is an ionic bond, forming a thermally non-reversible gel.

However, the use of a chelating agent, such as Ethylenediaminetetraacetic acid (EDTA), capable of diffusing in the gel meshes and chelating Ca^{2+} ions, provide an efficient dissolution

protocol for such gels. The ionic gelation of alginate is an interesting feature that will be faced in **chapter 4**, where alginate will be used as a biocompatible sacrificial microcarrier for A549 cell lines.

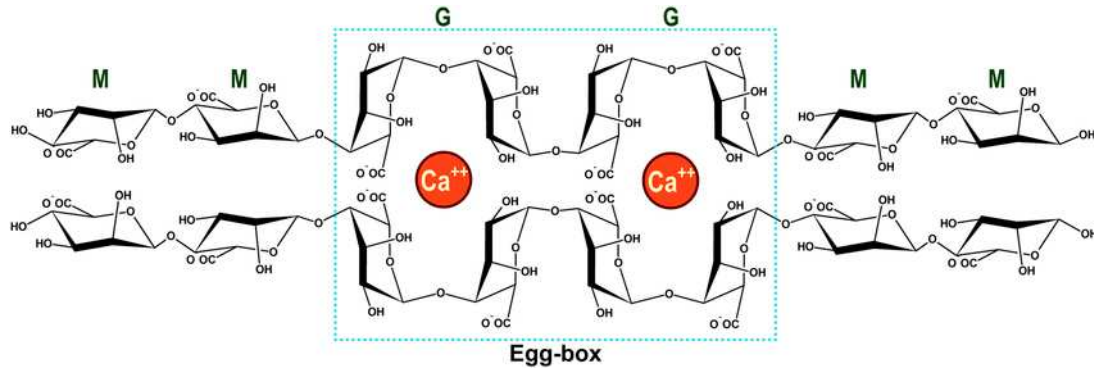


Figure 1.10: Schematic of "Egg-box" structure in alginate hydrogel crosslinked with calcium ions (*Adapted from H.R. Lee, et al. Immobilization of planktonic algal spores by inkjet printing. (2019) Scientific Reports.*) [60]

An interesting and pioneering work by *Huang et al.* tried to take advantage in LTE of alginate microbeads dissolution by EDTA. It was possible to use them as sacrificial mold to obtain a hollow alveolar 3D structure that was able to mimic distal airways morphology. Then, cell seeding was performed using an A549 cell line suspension after dissolution, in order to obtain a final 3D platform potentially useful for *in vitro* studies [61].

Table 1.3: Main properties of most commonly used natural polymers in STE.

Polymer	Main Advantages	Main Drawbacks	Ref.
Collagen (Col)	ECM component, High integrity, Highly bioresorbable	Lack of rigidity, Rapid degradation	[62, 46, 47, 48, 49]
Hyaluronic acid (HA)	ECM component, Good hydration, Good cells adhesion, Angiogenesis	Poor mechanical strength, Rapid degradation	[63, 64, 65, 66, 67, 68]
Chitosan (CH)	Similar to GAGs, Antibacterial	Low mechanical properties Solubility at acid pH, Polycationic properties Hemostatic properties,	[69] [70]
Heparin	Functionalizable, Anticoagulation, Anti-inflammatory	Poor cellular infiltration	[71, 59]
Alginate	High functionality, Chelating ability, pH-sensitive	Limited <i>in vivo</i> degradation, Poor cell adhesion, Mechanically weak, Poor printability	[72, 73, 60, 61]

1.3 Stem cells therapy for tissue engineering

1.3.1 Stem cells: properties and function

Stem cells (SC) are a class of undifferentiated⁶ cells that can multiply (self-renew) and differentiate into different cells within the human body, giving them enormous potential for use in regenerative medicine [75] (**Figure 1.11**). Thanks to their plasticity -in fact- SC are already used in numerous therapeutic applications, in particular for cell replacement therapy for many diseases, especially of the degenerative type.

All stem cells share some **common properties**:

- They can divide infinite times, differently from differentiated cells (e.g. blood cells, muscular cells, neurons, etc.), all losing replication capabilities (i.e. becoming senescent after 20-40 duplications). Now, since this property seems to be shared with neoplastic cells, this suggested a direct link between cancer stem cells;
- They are not terminally differentiated, as they did not reach the end of differentiative line yet;
- After a division two cells are born and they can be different (i.e. one keeps stemness tracts, the other faces a differentiative path) or identical (i.e. both of them keep stemness tracts, or differentiate). Therefore, what happens is both that the stem cell population is maintained (often referred to as *self-renewal*) and that differentiated cells are produced.

Moreover, a **stem cells classification** can be done according to two criteria, or based on their differentiative potential, or based on their origin:

I. Based on their differentiative degree, can be divided into:

- *Totipotent* stem cells: those able to form not only the whole organism but also some extra-embryonic structures;
- *Pluripotent* stem cells: those able to form all the organism's cells;

⁶Differentiation is defined as "the sum of the processes whereby apparently indifferent or unspecialized cells, tissues, and structures attain their adult form and function" by *Merriam-Webster Dictionary* [74].

- *Multipotent* stem cells: those capable of giving rise to several different types of differentiated cells;
- *Unipotent* stem cells: those giving rise only to a specific cellular differentiated type;

II. Based on their origin, can be divided into:

- *Embryonic* stem cells (ESCs): are pluripotent cells derived from the inner cell mass of the blastocyst, that can be grown and proliferated as undifferentiated cell lines;
- *Fetal* stem cells: those present in the fetus after gastrulation; they are multipotent cells capable of generating the various cell types present in the adult individual. Also stem cells coming from blood vessels of the umbilical cord are called "fetal";
- *Adult* stem cells: multipotent, oligopotent and unipotent cells that represent a minority subpopulation and are found in *stem niches*⁷. In addition, differentiated cells together with adult stem cells are the living component of all biological tissues are about 220 different types.

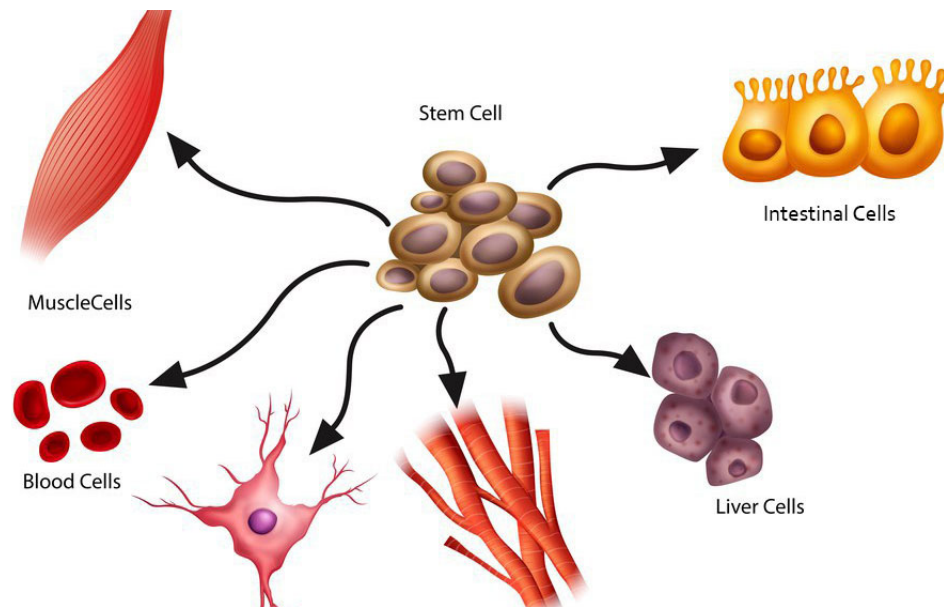


Figure 1.11: Stem cells possible post-differentiation outcomes.

⁷A microenvironment made up of the cellular elements surrounding the stem cells, of metabolites and of signals produced in the microenvironment for the maintenance of stemness [75]

Now, the possibility of growing differentiated cells of tissues such as skin, cartilage, vascular wall, lung tissue, etc. in the laboratory it presupposes the obtaining of this from the native tissue, their expansion in culture and their seeding and growth on a scaffold [76]. In particular, there are two possibilities: either you start from the patient's differentiated cell and induce their proliferation *in vitro*, or you can start from progenitor or stem cells and induce a certain degree of differentiation.

While in first case cells face a dedifferentiation, depending on chemical or physical conditions they are facing -it is then crucial to understand what are the transformations of the cellular phenotype during preparation in the laboratory. On the other hand, in case progenitor or stem cells are used, molecular mechanism of differentiation must be known -and that is even harder [77].

Clearly, cell implantation can take place once the cellular source has been defined. Actually, different cellular sources are available, each of which shows intrinsic advantages and disadvantages [78]:

- *Autologous* cell source: the donor and the recipient coincide (they are the same person). A clear advantage is, since the cellular portion of the implant cannot be rejected by the organism. On the other hand, this kind of systems shows limited availability and limited expandability;
- *Allogeneic* cell source: when the donor and the recipient are of the same species (in our case *Homo Sapiens*). This time in the face of greater availability and the possibility of industrialization there is a poor immune compatibility.
- *Syngeneic* cell source: the donor is genetically identical to the recipient this solution provides immunocompatibility (**Figure 1.12**) and a solution easy to be industrialized, with a clearly reduced availability only to cloned organisms;
- *Xenogeneic* cell source: the donor and the recipient are of different species. Obviously in the face of more than enough availability and ease in industrialization there is a serious risk of acute rejection, as well as zoonoses⁸ from endogenous viruses (e.g.HIV) or retroviruses (e.g.PERV).

⁸Defined as "an infection or disease that is transmissible from animals to humans under natural conditions" by *Merriam-Webster Dictionary* [79].

Once cellularized scaffolds are implanted, the body's response takes place: the reactions carried out by the cell surrounding the implant are fundamental for the achievement of the latter's objective or to determine its loss of functionality [80]. In fact, a healthy organism is an organism that responds through immune system to stimuli such as the insertion of a foreign body.

In the more specific case of an implant that uses autologous cells, which implies a complete acceptability of the cellular components it is however possible to assist has a reaction against the implant such as an excessive production of fibroblasts that can generate a fibrotic tissue around the implants themselves.

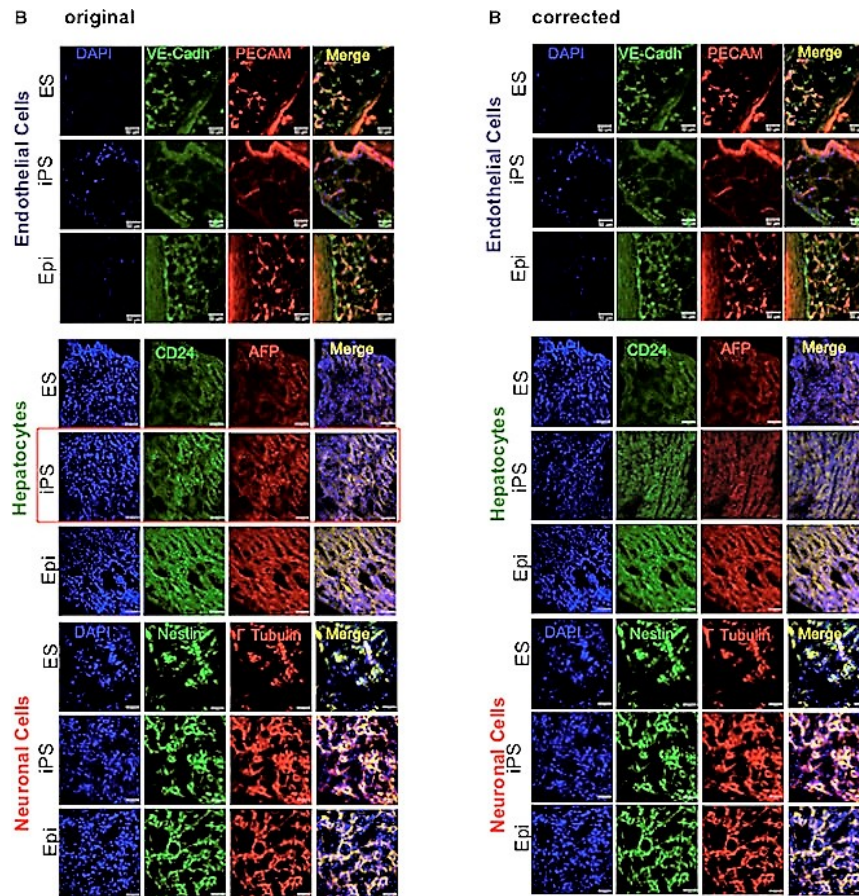


Figure 1.12: Differentiated Cells Derived from Syngeneic iPSCs (induced Pluripotent Stem Cells) are not rejected *in vivo* (original vs corrected). (Adapted from Guha, Prajna, et al. "Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells." *Cell stem cell* 12.4 (2013)) [81].

1.3.2 Stem cells for regenerative medicine

In literature, mainly type of stem cells are used for lung tissue repair: multipotent stem cells, such as MSCs, while pluripotent, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are mainly used for other soft tissue engineering applications.

Embryonic stem cells (ESCs)

Embryonic stem cells, being pluripotent stem cells, can differentiate into all type of tissues such as ectoderm, endoderm and mesoderm-derived tissue. Therefore, ESCs are obtained from firstly stage embryo; thus, there is a severe risk of transformation should consider for this source and hence, due to ethical problems, their usage is limited. In addition, some difficulties in their differentiation in the exact cell line.

Induced pluripotent stem cells (iPSCs)

In order to overcome ethical issues, iPSCs have recently drawn attention: basically, they are reprogrammed differentiated cells -like fibroblast- to embryo state. Their recent use involves some quite time-consuming procedures (i.e. extractions of fibroblast or chondrocyte from patients, reprogramming re-differentiating and implanting); moreover, an uniform differentiation can be difficultly achieved.

Mesenchymal stem cells (MSCs)

Firstly, they were extracted from bone marrow but nowadays they can be obtained from multiple tissues such as adipose tissue, umbilical cord, peripheral blood, trabecular bone, periosteum, lung, placenta, etc. and between them bone marrow and adipose-derived stem cells are the most used.

Their usage is also due to the fact that once hydrogels are put in the load bearing site, they cannot remain in the long term, since they may be degraded. Therefore, MSCs within the hydrogel will help hydrogel replacement by synthesizing the new, thus replacing the degraded biomaterial *in situ* [82].

1.3.3 Stem cells for lung tissue engineering

As discussed in subsection 1.1.2, current clinical therapies for lung injuries, provide for the use of MSCs, as they have the ability to differentiate into alveolar cells, lung epithelial cells, vascular and endothelial cells. The sources of MSCs include bone marrow (BMMSCs), umbilical cord tissue (Wharton's Jelly derived-WJMSCs), adipose tissue (ATMSCs), umbilical cord blood (UCMSCs), and menstrual blood (MBMSCs) [83].

As for BMMSCs, they would display a limited proliferative effect and a low quality when derived from aged or diseased donor. Instead, WJMSCs are very promising cells because the source is quite available and collection of the cells is noninvasive. Moreover, WJMSCs have compatible surface markers and cellular characteristics with BMMSCs and ATMSCs [84], they have better capacity of proliferation than BMMSCs and there has been no tumorigenicity reported up to date.

MSCs have potential beneficial effects in some respiratory models such as bleomycin-induced lung injury, abdominal sepsis and ventilator induced lung injury. With intravenous injection, MSCs can accumulate in the lung-injured regions, so that damaged lung releases some cytokines to attract MSCs and promote lung regeneration. However, in most cases, MSCs infusion provides a very low engraftment rate, assigned to what are termed paracrine effects [83].

Because of such interesting properties, attention has been brought to both understanding and exploiting their paracrine and other properties, regardless of their differentiation abilities. This is particularly important for lung diseases as well as destructive diseases with ongoing pulmonary and systemic inflammation even after insult cessation [85].

Although all the properties of MSC paracrine activities are yet to be determined, a partial list of actions includes the ability to sense local inflammatory environments through expression and activation of damage- and pathogen-associated molecular pattern receptors such as the Toll-like receptors.

The MSCs then release a spectrum of anti-inflammatory and other ostensibly reparative mediators including anti-inflammatory cytokines, antibacterial peptides, proangiogenic factors,

and extracellular vesicle particles that can affect lung epithelial cells as well as resident inflammatory and immune cells including alveolar macrophages.

Mitochondrial transfer from MSCs to damaged lung epithelial cells has also been detected. These mechanisms have been observed in in vitro studies in which exposure to MSCs or their secreted products affects lymphocyte proliferation and function as well as macrophage phenotype and activation. Accordingly, there have been a growing number of publications in which systemic or intratracheal MSC administration results in amelioration of injury in a range of preclinical lung disease models in rodents, larger animals (i.e. sheeps and pigs), and explanted human lungs [86].

It must be emphasized that, although there may be effects of MSCs on existing endogenous progenitor cells in the lung, current paradigms of MSCs actions in both preclinical models of chronic lung diseases such as COPD and in clinical trials focus on anti-inflammatory paracrine actions rather than structural repair. MSCs reside in many tissues, most notably in bone marrow and adipose, but have also been identified in the lung. After systemic administration, MSCs lodge in the lungs as the first major capillary bed which is encountered, predominantly through cell surface integrin actions which are still being defined.

Once in the lung, the cells do not completely engraft but rather release a range of soluble mediators and may also have some direct cell–cell interactions with resident pulmonary epithelial or vascular endothelial cells. Available data demonstrate that the MSCs are then cleared from the lungs over a 1- to 3-day period. Similar observations have been made after direct airway (i.e. intratracheal) MSCs administration. Clearance mechanisms are still being studied, but they include phagocytosis/efferocytosis by alveolar macrophages, induction of apoptosis, and other mechanisms [86].

1.4 Biomimetic approach to lung tissue engineering

1.4.1 The study of extracellular matrix

As explained in **subsection 1.1.1** in general the ECM is a substantial part of the volume of most of the tissues in humans, in long part filled with an intricate network of macromolecules that make up the extracellular matrix. The matrix comprises a variety of locally secreted proteins and polysaccharides which aggregate in a network organized in a compact manner and connected with the surface of the cell that produced them.

At the cellular level (10 μm) the cell is commonly associated with a control volume of ECM. In particular, there are specific connective tissues (e.g. tendons, dermis, cartilage) which can be modeled (on the scale of about 1 cm) as aggregates of ECM of relatively homogeneous composition and specific architecture [43].

ECM -especially lung's ECM- is mainly made up of different types of collagen, non-collagenous glycoproteins, hyaluronan and proteoglycans and serves as a scaffold, as well as a reservoir for growth factors and cytokines. In general, ECM is the extracellular part of multicellular structure (e.g. organisms, tissues, and biofilms) that typically provides structural and biochemical support to the surrounding cells [87]. Specifically, ECM role is mainly:

- Mechanical support for biomechanical functions and cell anchoring;
- Regulation and control of the 'traffic' of fluids, molecules, and nutrients;
- Provides a structural substrate for cell growth and remodeling;
- Reserve of soluble regulators.

In humans, the composition of the ECM can be simplistically reduced to a combination of water, proteins and polysaccharides: these three compartments are precisely balanced reflecting functional requirements of the specific tissues.

Obviously, any bio-engineered tissue should mimic in its composition -as well as in its structural and functional properties- the native tissue that it is going to replace. To this reason, any substrate produced for lung tissue engineering, whether it is meant for regenerative medicine

purpose, as a carrier or as a platform for bio-engineering models, has to possess specific properties that reflect the role of the native tissue’s ECM (as explained also in **subsection 1.1.1**).

1.4.2 The use of lung 3D constructs versus 2D cell culture and *in vivo* models

As already mentioned, chronic and acute diseases present significant challenges in clinical management, as neither asthma nor COPD can be cured; instead, palliative treatments that aim to alleviate symptoms, prevent further deterioration, and enhance quality of life are employed [88].

To date, even though experimental solutions do exist, their level of screening is not sufficient for further applications in clinical trials. For this reason, to tackle the growing challenges posed by lung diseases, the necessity for advanced *in vitro* models that can accurately replicate the lung microenvironment has become increasingly evident (**Figure 1.13**).

Conventional 2D cell culture setups are insufficient in replicating the intricate structural and functional aspects of the lung environment *in vivo*, not only from the point of view of cell-to-cell but also cell-to-matrix interactions. While basic single-cell systems offer valuable information about cellular responses, human models provide a more comprehensive understanding due to their ability to mimic natural interactions between cells or tissues.

As a matter of fact, these restrictions impede our comprehension of important cellular communications and the physiological mechanisms at play, like how cells interact with their surroundings and the relationships between different cell groups. The transfer of laboratory discoveries into practical treatments is difficult when lacking a three-dimensional structure and accurate co-location of various cell types, causing a gap in knowledge that hinders advancements in successful therapies [89].

In response to these challenges, alternative testing platforms have been explored. *In vivo* models, while they can provide comprehensive insights into physiological responses and inter-organ communication, raise ethical concerns and often require extended time frames for experimentation. Furthermore, these models may struggle to accurately replicate human-specific

disease mechanisms due to inherent physiological differences among species [90, 91].

On the other hand, lung-on-chip (LOC) technologies have emerged as promising systems capable of simulating dynamic flow conditions and biomechanical forces akin to those experienced in the lung. One of their main use is recreating the alveolocapillary barrier, that is the most commonly used platform to study nanosystems administrations and effect on alveoli's epithelial barrier [92, 93]. However, due to their nature, LOCs frequently lack the architectural and cellular complexity -as sometimes they are bidimensional layers of an ECM-like matrix without architecture- necessary for reliable therapeutic testing, limiting their predictive capabilities.

Recent advancements in the development of 3D models have the potential to revolutionize regenerative medicine and lung tissue engineering. Biomimetic scaffolds composed of natural polymers such as collagen and hyaluronic acid, are widely used to replicate the native lung environment more faithfully. These scaffolds facilitate cellular organization and differentiation while promoting interactions that closely resemble those of native lung cells.

In particular, in pulmonary research, various cell lines have been extensively utilized to model distal airways, each with distinct advantages and limitations. A549 cells, derived from human lung carcinoma, are among the most commonly used cell lines due to their ability to proliferate rapidly and their established protocols for differentiation into alveolar epithelial-like cells. However, even though their cancerous origin raises concerns, their behavior when seeded in 3D matrixes seems to show a more relaxed tumoric phenotype [89].

More in detail, their consistent metabolic and transport characteristics akin to ATII, allow researchers to accurately study cellular functions [94]. The uniform genetic makeup of A549 cells ensures consistency in tests, and their capacity for large-scale culturing enables thorough experimentation. Furthermore, they have the ability to create cohesive single layers that show the standard shape of ATII, with lamellar bodies present, and they have the capability to produce inducible P450 enzymes (IA1 and IIB6) that parallel the functioning of ATII *in vivo* [95, 96].

Nevertheless, there are drawbacks associated with the use of A549 cells; the monolayers they create are permeable because they do not have tight junctions, resulting in weakened barrier integrity. This leads to extremely low trans-epithelial electrical resistance (TEER) values, which complicates the accurate measurement of the transportation of small molecules with low molecular weight. Furthermore, the lack of genetic and phenotypic diversity could restrict the capacity to replicate the variations seen in primary lung cells.

Conversely, human bronchial epithelial cells (HBECs) offer a more physiologically relevant model, as they are derived from normal human tissues. These cells exhibit closer similarity to native epithelial function and structure, but their limited lifespan in culture can pose challenges for extended studies, together with lower cell obtaining yield via bronchoscopy and a high potential for microbial contamination. [97, 98, 99].

Same way, primary ATII cells are also employed, providing a direct representation of alveolar epithelium; they are widely regarded as the gold standard for comparing other cell types due to their ability to accurately replicate *in vivo* behavior and their metabolic activity [100]. In particular, their controllable cell division facilitates model validation, and they are capable of undergoing TGF-1-induced epithelial–mesenchymal transition in culture, reflecting crucial cellular processes in pulmonary pathology [101].

Additionally, ATII cells possess the necessary cell surface receptors for effective signaling, which is vital for studying cellular interactions in the lung microenvironment. However, the isolation of ATII cells is time-consuming and presents a high risk for bacterial or fungal contamination, leading to concerns about purity. Furthermore, achieving 100% pure isolation is often unattainable, and the financial cost associated with isolation reagents can be significant. Once isolated, these cells cannot be passaged repeatedly, and their growth can be dependent on maintaining an adequate seeding density, complicating experimental design and scalability [102].

Overall, the choice of cell line for modeling distal airways is critical, as it directly influences the translational potential of research findings, necessitating a careful consideration of each line’s inherent pros and cons.

For this reason, despite these advancements, significant gaps remain in current models, particularly concerning the co-culture of fibroblasts and epithelial cells—critical for accurately mimicking lung microenvironments. A thorough understanding of the dynamic interplay between ECM components, three-dimensional architecture, and the spatial colocalization of various cell types is essential for the effective translation of experimental findings into clinical applications. Thus, there is an urgent need to develop advanced models that bridge the divide between *in vitro* and *in vivo* studies, ultimately leading to more effective treatments for respiratory diseases.

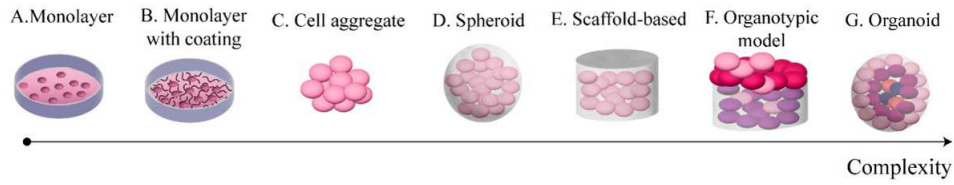


Figure 1.13: Progression in cell culture models complexity, from bidimensional to fully developed three-dimensional culture systems. (A) Simple 2D monolayer cultures on polystyrene surfaces. (B) Protein-coated monolayers with substrates like collagen or fibronectin. (C) Basic 3D cell aggregates formed by cellular adhesion. (D) Spheroids with external support enhancing structural stability. (E) Scaffold-based cultures introducing ECM surrogates. (F) Organotypic models replicating tissue structure and function with multiple cell types and support materials. (G) Organoids representing highly complex organ-like structures with organization mimicking native tissues. (Adapted from Cortesi, Marilisa, et al., *PeerJ* 12 (2024): e17603.) [103]

Aim of the PhD thesis

In biomedical applications, the criteria for selecting the materials as biomaterials are based on their specific chemistry, molecular weight, solubility, shape and structure, hydrophilicity/hydrophobicity, lubricancy, surface energy, water absorption degradation, and erosion mechanism.

More in detail, a significant contributions of natural compounds -especially natural polymers- is found in tissue engineering. They particularly find a wide range of applications thanks to the fact that they can be engineered into micro- and nanostructured systems, enabling their use not only in the field of delivery of therapeutic agents, but also tissue scaffolds for regenerative medicine or biomedical research purpose.

Novel and natural polymeric strategies are oriented toward improvement of various treatments. In natural polymeric approaches, encapsulation of active cells and drugs in scaffolds and their target on specific sites is done in a controlled manner.

Natural polymers also often possess good cytocompatibility and other advantages over synthetic polymers making them a popular option for tissue engineering scaffolding applications. Biopolymers, with their capacity to serve as carriers and tissue engineering scaffolds, have a significant role in the area of regenerative medicine [104].

In particular, soft tissue engineering strategies targeting restoration of volume loss have inherent critical challenges as they relate to the problem of restoration of defects with a high volume to surface ratio. In order to help the regeneration of the injured tissue or to substitute damaged tissue and finally to repair the functionality of these tissues or organs, tissue engineering uses combinations of cells, bioactive factors, and supporting structures [105].

As a matter of fact, natural compounds have shown great potential for use as a substrate for tissue engineering. Their biomimetic nature allow to mimic the extracellular matrix by replacing the damaged tissues ensuring a substrate that is as close as possible to the real physiology of the tissues. Furthermore, other characteristics are good biocompatibility, biomimetic, swellable properties, and enviable physical characteristics that allow permeability for oxygen, nutrients, and water-soluble metabolites, fundamental in creating functional scaffolds.

Lung diseases are countless and due to a plethora of reasons. Probably, the most common and invalidating ones are chronic pulmonary diseases: COPD, asthma and pulmonary bronchodysplasia are few examples. In particular, COPD is the third leading cause of death worldwide, causing 3.23 million deaths in 2019, 80% of which occurred in low- and middle-income countries [106].

Additionally, acute respiratory distress syndrome (ARDS) is another serious condition associated with the burden of lung disease, typically stemming from significant lung injury or issues from infections, such as viral pathogens like SARS-CoV-2 [107]. Bacterial infections are also a common trigger for ARDS, as they can lead to quick development of extensive inflammation in the lungs and consequent damage, especially in individuals with underlying chronic respiratory diseases [108].

Therefore, this pathology results in respiratory failure and high death rates, particularly in individuals with pre-existing chronic lung disorders such as COPD. Although mainly impacting people in intensive care units, ARDS worsens the worldwide impact of lung disease, underscoring the urgent call for new treatment approaches and preventive measures for all populations.

Currently there are no effective treatments following alveolar damage for these pulmonary pathologies and cardiopulmonary bypass or transplantation are still solutions restricted to extremely serious cases, and for sure not affordable for everyone. New frontiers for lung regeneration are found among cellular based lung tissue engineered scaffolds, trying to mimic the native tissue structure and composition.

Aside from that, also bacterial lung infections pose a significant risk, particularly due to their ability to induce acute lung injuries (ALI) characterized by severe inflammation, alveolar

damage, and compromised respiratory function. ALI triggered by infections can lead to acute respiratory distress syndrome (ARDS), often requiring intensive medical intervention.

Now, those bacterial infections are primarily caused by pathogens such as *S.pneumoniae*, *H. influenzae*, and *P. aeruginosa*, which can lead to ALI and subsequent ARDS. In patients with COPD, the risk of such infections is notably heightened due to pre-existing lung inflammation and compromised respiratory function. The aberrant remodeling of the ECM associated with COPD creates a favorable environment for pathogen colonization, exacerbating inflammation and further impairing lung function.

As a consequence, these infections not only trigger ALI but also worsen the underlying COPD symptoms, creating a vicious cycle that complicates treatment and significantly impacts patient outcomes [109]. Understanding the interplay between these pathogens and the pathological changes in lung tissue is essential for developing targeted therapeutic strategies to manage both infections and chronic respiratory conditions effectively.

Current treatments involve antibiotics and supportive care, such as mechanical ventilation for severe cases [110]. However, antibiotic resistance remains a critical challenge [111], reducing treatment efficacy and necessitating prolonged or more aggressive interventions, which carry risks of lung injury from ventilator use and limited efficacy in advanced infections. Novel therapeutic approaches are being investigated, but effective and targeted solutions for resistant infections are still needed.

In addition to that, a major limitation in studying experimental lung therapies is the discrepancy between *in vitro* and *in vivo* models, which often leads to failures in clinical translation. Conventional 2D cultures lack the structural complexity and cellular interactions of lung tissue, resulting in poor predictive value. To address this, 3D models have been developed as more realistic platforms, designed to better mimic lung tissue architecture and microenvironment [112]. However, even advanced 3D models often fall short of capturing the full spectrum of *in vivo* conditions. Bridging this gap with improved, biomimetic 3D lung models is essential for enhancing the accuracy of preclinical studies and increasing the success of experimental therapies in clinical applications [113].

The purpose of this PhD thesis was, therefore, the study of substrates based on natu-

ral compounds for lung tissue engineering applications. In particular, the research activities presented here concern the use of natural compounds in the field of substrates engineering for regenerative medicine application, against both acute and chronic damages occurring also during bacterial infections. Besides, they were also employed for the design of 3D constructs, that can be used to model lung tissue.

Among these natural compounds, mainly natural polymers such as hyaluronic acid and collagen have been used in a biomimetic approach, being a fundamental components of the extracellular matrix of lung tissues.

Briefly, initially we addressed the design and investigation of a micro-sized biomimetic biphasic stem cells delivery system. In particular, a mobile stem niche composed of microspheres made of self-assembled type I collagen (mCOLLs) and suspended in a low molecular weight hyaluronic acid (HA) solution.

In particular, to optimize such delivery system, dimensional distribution analysis of mCOLLs in both regular and HA-enriched media, as well as injectability studies, were conducted. Additionally, the systems' capacity to act as reservoirs for viable MSCs was assessed, confirmed through morphological analysis. MSC differentiation into ATII cells was also evaluated in HA-enriched medium, highlighting the system's potential as a pulmonary stem niche.

Then, the second part of the thesis has been dealing with the aforementioned problem of bacterial lung infections and the multi-drug resistance issue. To this end, nano-sized folic acid-based carbon dots (FA-CDs) were synthesized using a one-step temperature-controlled microwave-assisted method, testing three synthesis temperatures (130 °C, 170 °C, and 185 °C) to examine their effect on physicochemical properties and biological response of CDs.

FT-IR and UV-vis spectra confirmed new chemical bonds and surface functionalization, showing the presence of carboxyl and amino groups. Urea played a key role as an additional nitrogen source for surface modification. Biocompatibility tests indicated a preferable synthesis temperature, as well as an optimal concentration range (300-500 $\mu\text{g/ml}$) after 7 days of incubation with normal human lung fibroblasts (NHLFs). Additionally, such CDs demonstrated strong antimicrobial activity, inhibiting gram-positive bacteria like *Staphylococcus aureus*.

Eventually, it became evident the need of a proper biomimetic 3D substrate that could better mimic the complex architecture and cell population distribution of distal airways. Therefore, it was built a micro-sized 3D alveolar structure capable of mimicking, not only the 3D porous structure of human alveoli, but also the main characteristic of epithelial and stromal phenotypes of cells, focusing for the first time on their specific co-localization. To this aim, different natural polymers such as HA, collagen and alginate were employed in a series of bulk micro-fabrication techniques.

Then, such systems were optimized in terms of mechanical properties and cells distribution and then characterized from a biological and immunohistochemical point of view, showing such systems as a promising and complete platform to study healthy and fibrotic response of distal airways in future studies, serving also as a substrate for future drug screenings.

In fact, a full 3D model was constructed that can replicate both the ECM elements and the alveolar structure. A functional alveolar model was constructed in this system by achieving precise co-localization of stromal and epithelial cell populations, which could secrete Surfactant protein-C (SP-C) and create barriers with tight junctions.

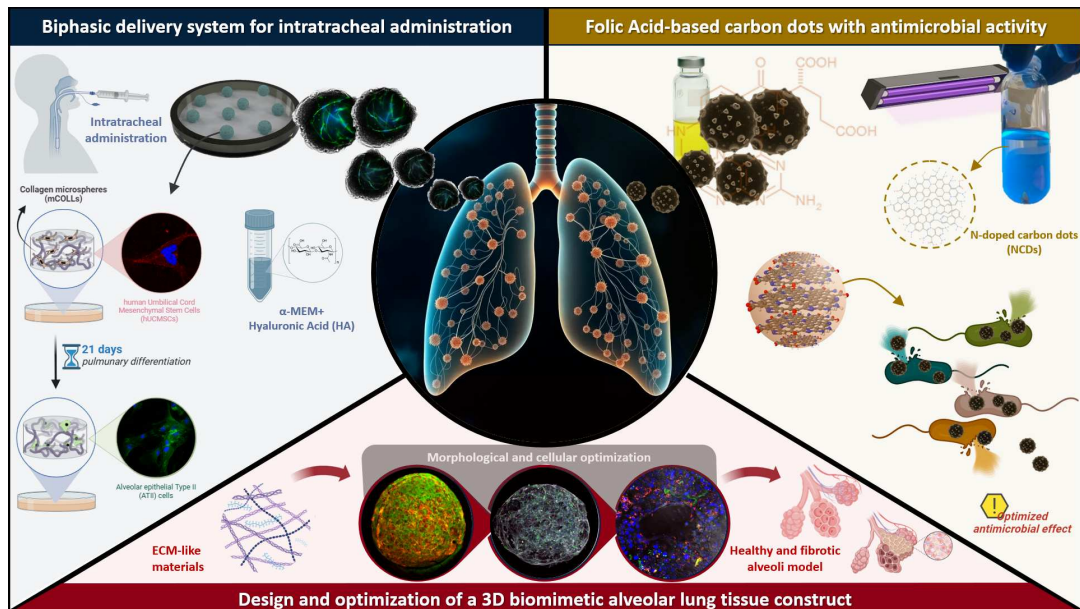


Figure 1.14: Scheme of the study conducted in this PhD work.

Chapter 2

Collagen-HA biphasic stem niche delivery systems for pulmonary differentiation

Contents

2.1	Introduction	47
2.2	Materials and methods	51
2.2.1	Materials	51
2.2.2	HA solution rheological characterization	51
2.2.3	Cell culture and mCOLLs preparation	52
2.2.4	mCOLLs diameter characterization and injectability by rheological characterization	54
2.2.5	mCOLLs viability and morphology	56
2.2.6	ATII expression study	57
2.2.7	Statistical analysis	57
2.3	Results and discussion	58
2.3.1	HA solution rheological characterization	58
2.3.2	MSCs stemness phenotype evaluation by flow cytometry	60
2.3.3	mCOLLs diameter characterization	60

2.3.4	Rheological characterization	65
2.3.5	Cell delivery of MSCs from mCOLLs	67
2.4	Conclusions	69

2.1 Introduction

Nowadays, lung-related pathologies, both acute and chronic, remain among the most diffused leading causes of death, and disability worldwide [1, 2]. Particularly, the most common pathologies include asthma, COPD, ARDS, BPD of the preterm infants and, lately, SARS-CoV-2 pneumonia stand out. One of the most complex and problematic aspects of those pathologies is the associated dysregulation in immune responses and the following repair processes and remodelling of the lung extracellular matrix (ECM), which result anomalous [3].

Therefore, the absence of resolution of the pulmonary lesions lead to a decrease both at the numerical and functional level of the fundamental unit of the lungs, the alveoli, and principally of the ATII cells. To date, beyond palliative procedures to relieve patient's symptoms, there are no other effective treatments for lung injured epithelia. The cell therapy with intratracheal administration of MSCs was proved to be an highly potential treatment in lung regenerative medicine based on preclinical and clinical studies [4, 5, 6]. Indeed, thanks to stem cells' ability to differentiate in ATII cells according to specific stimuli of the niche/tissue environment, the repopulation of the compromised areas could be possible. Furthermore, through paracrine mechanisms they would help the release of pro-angiogenic and anti-apoptotic factors, with beneficial effects on lung lesions [7, 8].

Besides, pulmonary diseases are associated to the failure of lung structure and the consequent preclusion of the correct homing of MSCs delivered. Various hydrogels, both of synthetic and natural origin, have been investigated in the literature for the delivery of MSCs. For example, injectable scaffolds with different chemical functional groups were designed by introducing one equivalent of α -cyclodextrin (α -CD) carboxylate and α -CD phosphate along poly(ethylene glycol)-poly(l-alanine) (PEG-L-PA) block copolymers to control osteochondral differentiation of MSCs [9]. Generally, the main advantages in the use of hydrogels are mainly associated with the ease of cells encapsulation, moreover, due to the intrinsic property of retaining water, hydrogels guarantee a greater exchange of oxygen and nutrients for the cells. Furthermore, if desired, these can be finely tuned to achieve effective differentiation of stem cells.

Even so, some limitations concern i.e. the low mechanical properties of the hydrogels, and inherent immunogenic responses with biological origins. To improve the modulus of such

hydrogels is often used the incorporation of chemical modification with synthetic polymers that can have detrimental effects on cells [10]. Currently, existing methods for delivering cells include suspending them into hydrogel or physiological solution for the injection [11]. The low viscosity in a sol state, as well as allowing injectability, encourages cell survival following injection [12]. Nevertheless, the immediate loss of the majority of the injected cells as a result of the injection path backflow has been reported in the cells suspended in saline solution [13]. In Addition, several studies have expressed concerns about shear-induced cell death during syringe injection when utilizing hydrogel, due to cell damage from mechanical stress [14, 15].

These drawbacks result in a low level of cell engraftment after the delivery [16, 17]. Furthermore, the survival-apoptosis regulation of the MSCs are dependent to anchorage by physical interactions with ECM [18]. Thus, this lack can further lead to a low engraftment of post-injection survived cells and consequently a poor functional remodelling of the tissue [19]. In addition, the de-arranged ECM in lung diseases can strongly contribute to the poor cell attachment. Therefore, a delivery system shielding and creating a temporary home for the cells mimicking their natural niche, would be capable to improve the MSCs viability, the residence time at the target site, also participating actively in the regenerative remodelling pathway. The pulmonary niche is the place where the interplay between ECM and cells takes place, so that the remodelling processes and the consequent regeneration can occur.

As discussed in **subsection 1.1.1**, Lung ECM is a complex and dynamic network constituted by fibrous proteins, including collagen (Col), which play the most important structural factor in determining the elastic recovery during gas exchanges in lung [20]. Moreover, GAGs such as hyaluronic acid HA represent another key component in the ECM lung parenchyma, playing central roles both in water regulation in the interstitium [21] and in the stabilization of the surfactant proteins [22].

Furthermore, it has been determined that the exogenous HA administered by aerosol or tracheal instillation acts against inflammation, protecting the airways against the hyper-reactivity and remodelling of the bronchial and lung parenchyma [23, 24]. Moreover, our group previously found out that in bi-dimensional cell culture systems, low and medium Molecular Weight (MW) HA enriched media can increase the differentiation of MSCs to ATII cells capable of secreting pulmonary surfactant proteins compared the high MW HA [4, 25]. Commonly,

microencapsulation techniques have been investigated providing a platform for cell adhesion and a suitable substrate for tissue regeneration [18, 26, 27].

Nevertheless, although materials such as those derived from natural ECM demonstrate excellent biocompatibility and low immunogenicity, the detrimental effects of the crosslinking agents on the biological activities limit their use in tissue regeneration [28]. In this frame, the aim of this work has been to develop a biphasic stem cell biomimetic niches delivery systems capable to improve the viability and the differentiation ability of the MSCs in ATII cells. To this aim, physiological microenvironment constituent of the stem niche has been used. Collagen (COLL) microspheres encapsulating MSCs (mCOLLs), without any chemical crosslinker that could affect biocompatibility has been developed and incorporated in injectable HA solution. As a matter of fact, it is possible in such systems to maintain stemness or direct the differentiation towards a particular lineage by specific biochemical cues 29.

Previous studies from our group demonstrated that MSCs embedded in three-dimensional COLL matrix and incubating with lung media implemented by HA at different MW (Low, medium and High) improve the differentiation in ATII cells with a considerably increase of the surfactant proteins expression, without finding a dependence on the HA MW used [29]. However, it has been demonstrated in preclinical studies in mice that HA MW in a size range of about 67 and 215 kDa showed longer persistence in the lungs. Indeed, lung exposure appeared to be optimum in this size range due to the rapid absorption of HA <67 kDa and the poor lung penetration and mucociliary clearance of viscous solutions of HA > 215 kDa [30].

Thus in this work, mCOLL systems have been suspended in a solution of Low (L) MW HA using to specifically improve the differentiation of the MSCs in ATII cells (see **Figure 2.1**). Furthermore, the HA is a naturally occurring mucoadhesive polymer which are able to form entanglements or non-covalent bonds with the mucus covering epithelial tissues [31]. This feature can prolong the delivery system in vivo residence time, constituting the main advantage of the biphasic system. In this work, in order to optimize the delivery systems, a consistent statistical analysis has been performed on dimensional distribution of mCOLLs, both in culture medium then in medium enriched with HA, as well as a study on the injectability of these systems, comparing the clinical practise currently applied in cell therapy.

Moreover, to study the delivery capability of such systems, the ability to act as a reservoir of viable MSCs was tested. All data were also confirmed in terms of morphological analysis by confocal microscopy. Eventually, it has further evaluated the MSCs differentiation in ATII cells when seeding mCOLLs in HA-enriched medium, to prove the strong potentiality of these systems as a pulmonary stem niche.

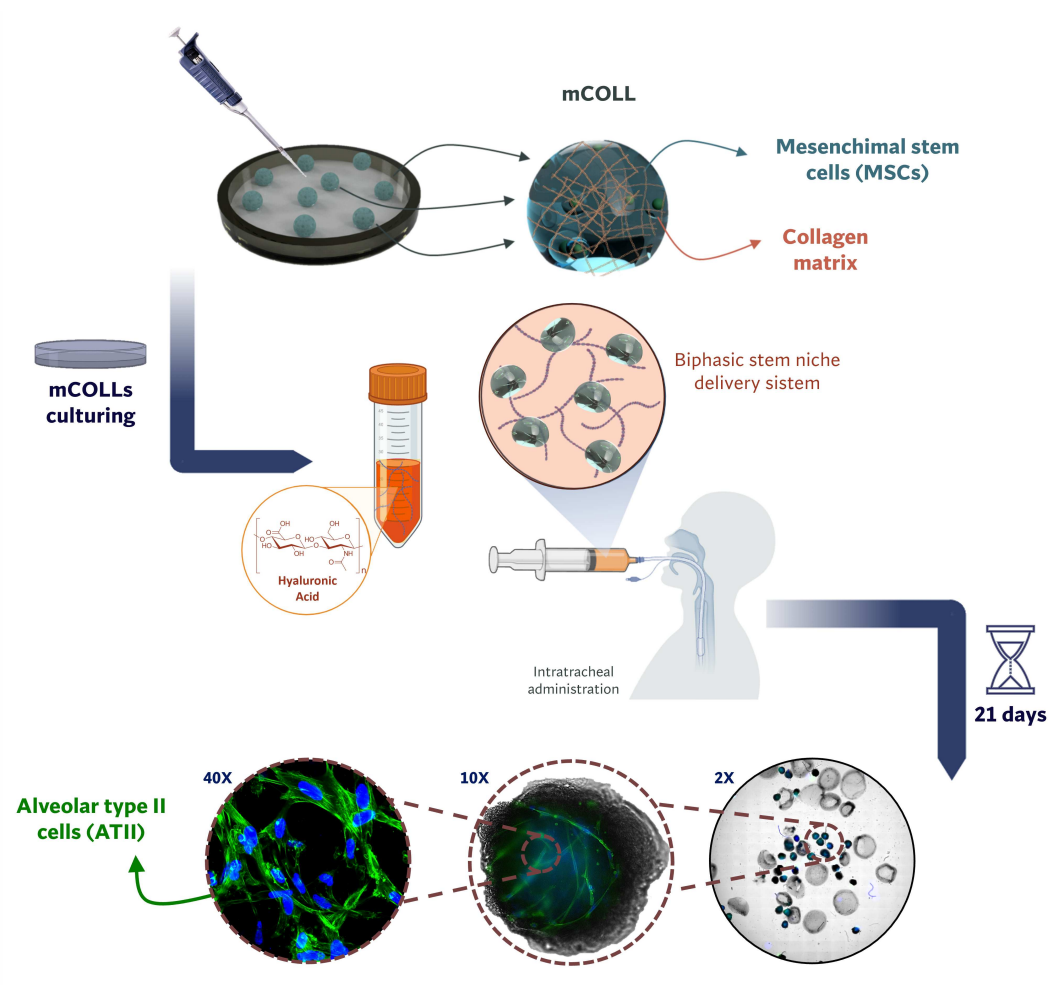


Figure 2.1: Scheme of the study. Collagen microspheres (mCOLL) embedding mesenchymal stem cells (MSCs) were suspended in a solution of low molecular weight Hyaluronic Acid (LMWHA) to specifically improve the differentiation of the MSCs in ATII cells. Moreover, the MSCs-delivery capability of such systems, was tested in terms of injectability for intratracheal administration and ability to act as a reservoir of viable MSCs.

2.2 Materials and methods

2.2.1 Materials

HA with a weight-average of low (L, 200 kDa), medium (M, 500 kDa) and high (H, 1435 kDa) molecular weight (MW)), were kindly provided by Altergon Italia s.r.l.. Phosphate buffer saline (PBS) tablets without calcium and magnesium were obtained from MP Biomedicals Inc. hUCMSCs cells were extracted from the Wharton jelly of the umbilical cord kindly gifted by Ospedale Evangelico Betania (Naples, Italy). Dulbecco's Modified Eagle Medium (DMEM) (Microgem Italy). Small Airway Epithelial Cell Growth Medium (SAGM) (Lonza C-41 24) and Fetal Bovine Serum (FBS) were purchased from Lonza (Basel, Switzerland). Eagle's Minimum Essential Medium (EMEM), Hyclone, (MA, USA). Penicillin, streptomycin (10,000 U/mL) from Invitrogen and Life Technologies (Carlsbad, CA, USA) were employed. Trypsin and Ethylenediaminetetraacetic acid (EDTA) were from HiMedia (Mumbai, India). Mitomycin C (MMC), Formalin, bovine serum albumin (BSA) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (USA). Pulmonary-associated surfactant protein C (SPC) rabbit antibody from Abcam. Fitch-conjugated anti-rabbit antibody (Millipore, Billerica, MA, USA). Human pulmonary surfactant associated protein A-B-C and D ELISA kits were obtained from Elabscience.

2.2.2 HA solution rheological characterization

In order to preliminarily study the behavior of different MW HA chains in solution, rheological tests were performed by means of a MARS rheometerTM III (TermoFisher Scientific, Waltham, MA, USA). The geometry used was Cone C60/1° CS L. Flow curves were conducted at 25° C on LMWHA, MMWHA and HMWHA solutions at different concentration ranges (0.125-40 mg/ml, 0.125-30 mg/ml and 0.125-11 mg/ml, respectively) in distilled water. The range of shear stresses varied from 0.01 to 1100 s⁻¹ depending on polymer MW and concentration to obtain a curve representative of their behavior. Each flow curve was repeated in triplicate and error-correlated data were fitted on OriginPro 2018 software. Ellis-fluid model was implemented to study the dependency of viscosity on the shear rate calculated with **Equation 2.1**:

$$\eta(\dot{\gamma}) = \frac{\eta_0}{1 + (\lambda \dot{\gamma})^{(n-1)}} \quad (2.1)$$

Where η_0 stands for the *zero-shear viscosity*, λ is a characteristic time constant representing the *relaxation time of the polymer solution*, and n is a *flux index*. Then the fitted values for zero-shear viscosity (accompanied by the fit uncertainties) were used to calculate the specific viscosities η_{sp} according to **Equation 2.2**:

$$\eta_{sp} = \frac{\eta_0 - \eta_s}{\eta_s} \quad (2.2)$$

Where η_s is the *solvent viscosity* (i.e. distilled water). Then, the specific viscosities have been plotted on a log-log graph as a function of concentration for all MWs and three weighted linear fits for each of these plots were performed. In order to understand the critical concentrations for polymer coils overlapping, these have been reported as a function of MW to obtain a phase-diagram useful for highlighting HA solution regimes at different MWs.

To assess the reaching of a semidilute entangled regime of HA solutions, a strain sweep was conducted on LMWHA, MMWHA and HMWHA solutions (40 mg/ml, 27 mg/ml and 10 mg/ml respectively) at 25°C and a frequency of 1 Hz, in a strain rate range from 0.1 to 50%. Then, linear regime was evaluated, and a frequency sweep was performed on same solutions at 25° C and 1% strain, investigating a frequency range from 0.003 to 20 Hz depending on the MW.

2.2.3 Cell culture and mCOLLs preparation

hUCMSCs cells were used at early passages (3–6). The cells were grown in a T-75 cell culture flask (Falcon, Italy), in Minimum essential medium with the alpha modification (α -MEM), supplemented with 10% FBS, 8 mM l-glutamine and antibiotics (penicillin G sodium 100 U/mL, streptomycin 100 U/mL), in a humidified and controlled atmosphere at 37 °C and 5% CO_2 . The medium was changed every 2–3 days. When confluent growth was reached, the cells were detached with 0.25% trypsin–EDTA solution and washed twice with PBS. The resulting cell suspensions were centrifuged (5 min, 1000 rpm; BRK55/10 Centrifuge by Centurion

Scientific Ltd., Chichester, UK).

To prepare mCOLLs, hUCMSCs at different passages were seeded into collagen solution (final concentration: 2.2 mg/ml). In particular for differentiation experiments P3 hUCMSCs were used, in order to better preserve differentiative capabilities. For viability assessments also P4 hUCMSCs could be used, while for other experiments (i.e. mCOLLs contraction studies, rheological behavior, etc.) P4 to P6 hUCMSCs were used, since no difference in experimental results were observed. Briefly, a type I collagen solution from bovine skin (80% v/v, 2.9 - 3.2 mg/ml, Sigma Aldrich), 0.1 M NaOH (10% v/v) and sterile PBS 10X (10% v/v) were mixed avoiding formation of bubbles, ensuring, adjusting pH of the final solution with few drops of collagen or NaOH solution ensuring a $pH \simeq 7.2$.

Once confluent cells growth was reached, as described above. Viable cells were counted using the TC20 automated Cell Counter (Biorad, Des Plaines, IL, USA). Trypsinized cells were suspended in culture medium at a density of 1×10^5 and 1×10^6 cells/ml and the pellet obtained by centrifugation was mixed with collagen solution. In order to produce the mCOLLs for the MSCs delivery, first, cut Parafilm® was placed over the lid of a 100 mm Petri dish and, after ensuring the correct adhesion to the bottom of the lid, the system was left for 10-15 min under a UV light to ensure complete sterilization.

The suspension of MSCs in collagen solution was pipetted through a P10 tip, forming 2.5 μ l droplets on the sterilized Parafilm®. Then, the system was incubated at 37°C and 5% CO_2 for 40 min to induce collagen fibrillation. The dish was incubated with culture medium on the bottom of the Petri, to create a saturated ambient, to avoid mCOLLs evaporation during fibrillation. Immediately after gelling, mCOLL on Parafilm® was detached with fresh culture medium. mCOLLs within cells were cultured in α -MEM, SAGM, sterile PBS 1X or the same medium enriched with LMWHA respectively depending of the tests. As for the samples in which cells were treated with MMC, the only difference in preparation takes place before cell seeding.

Briefly, MMC was added to the culture medium to a concentration of 10 μ g/ml as reported in literature [32] and cells were incubated at 37°C and 5% CO_2 for 1h. Then cells were gently washed with culture medium twice and with sterile PBS 1X twice, before usual

trypsinization and seeding at a density of 1×10^5 and 1×10^6 cells/ml.

2.2.4 mCOLLs diameter characterization and injectability by rheological characterization

Optical microscopy with a 4X objective was used to capture images of MMC treated and untreated mCOLLs at different days, up to full diameter stabilization (from day 0 to day 10). Then, acquired images were imported into ImageJ software (NIH, Bethesda, MD, USA) for post-processing analysis and quantification of mCOLLs diameter.

Briefly, a statistic was made by analysing 25 droplets per day or fraction and for each droplet three different diameters were measured. For each day of culture all the significant statistical parameters (first three quartiles and IQR, the interquartile range) were reported in terms of a boxplot using Matlab programming platform. All standard deviations seemed to be lower than 10% of the mean value.

Then standard deviations deriving from the measuring of three diameter for each droplet were plotted in terms of σ/σ_0 (with σ_0 being the standard deviation at day 0) was used as a parameter of the shape homogeneity. Normalization with respect to the standard deviation at day 0 was done in order to be independent on the operator error at pipetting. This normalized value was reported as a function of days of culture for different samples. Such characterization was conducted for two different seeding densities ($1 \cdot 10^5$ and $1 \cdot 10^6$ cells/ml) in medium.

The relative diameters D/D_0 (with D_0 being the diameter at day 0) were plotted for $1 \cdot 10^6$ cells/ml seeded mCOLLs cultured in α -MEM or α -MEM/HA and were fitted on Matlab by using as empirical law:

$$\frac{D(t)}{D_0} = D_\infty - (D_\infty - 1) \cdot \exp(-k \cdot t) \quad (2.3)$$

that takes into consideration only to parameters for fitting: D_∞ , which is the stabilized diameter and k , a constant that represents the rate of exponential decay. Eventually, the standardized residuals d_i for each measurement from the non-linear fit were considered (in the

plot the subscript i was omitted):

$$d_i = \frac{y_i - \hat{y}_i}{\sqrt{\frac{\sum_i (y_i - \hat{y}_i)^2}{n-p}}} \quad (2.4)$$

To further evaluate the goodness of the fit, also the reduced χ^2 parameter was considered:

$$Reduced \chi^2 = \frac{\sum_i (y_i - \hat{y}_i)^2}{n-p} \quad (2.5)$$

Where y_i represents the value of the measured experimental data, $\hat{y}_i$ the prediction of the model given the experimental condition, n the number of data and p the parameters in the model (in this case $p = 2$, since it is only a two parameters model).

In order to evaluate the injectability of the suspension of mCOLLs system for intratracheal administration, rheological tests were performed by means of a MARS rheometerTM III (TermoFisher Scientific, Waltham, MA, USA). The geometry used was Cone C60/1° CS L. In detail, firstly flow curves were conducted comparing the viscosities of PBS 1X and PBS 1X implemented with LMWHA (5 mg/ml), with and without mCOLLs in a range of shear stresses from 1 to 1000 s^{-1} . Data were fit using two different rheological models: a simple Newtonian model was used to fit the PBS control, and thus was used the following equation:

$$\tau(\dot{\gamma}) = \eta \dot{\gamma} \text{ , with constant } \eta \quad (2.6)$$

Where $\dot{\gamma}$ represent the shear rate, τ represent the shear stress and η represent the viscosity of the system. Instead for other samples containing HA, mCOLLs or both, the Cross-fluid model was used to model the dependency of viscosity on the shear rate:

$$\eta(\dot{\gamma}) = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1 + (k \dot{\gamma})^n} \quad (2.7)$$

Where η_0 represents the zero-shear viscosity, k is a characteristic time constant, and n is a flux index and η_∞ represent the terminal viscosity, the value of our interest, that was further plotted in terms of relative terminal viscosity (normalised with respect to the one of the PBS+cells system, representing the state of art).

2.2.5 mCOLLs viability and morphology

mCOLLs viability at different times was evaluated culturing them with α -MEM or α -MEM/HA (with 5 mg/ml of LMWHA). 35 mCOLLs/ well in triplicate (equivalent to 8.75×10^4 cell/ml) were placed on 24-wells (World Precision Instruments, Inc) cultured up to 1,4,7,10 days. The Alamar blue assay (AB) was performed by adding the reagent (at 10% v/v) to the samples and incubated at 37°C for 4 hours. AB is an indicator dye that incorporates an oxidation-reduction indicator that changes colour in response to the chemical reduction in the growth medium, resulting from cell viability. Reduced AB was withdrawn absorbance of the samples was measured using a spectrophotometer plate reader (Multilabel Counter, 1420 Victor, Perkin Elmer) at 570 nm and 600 nm. Data are expressed as the percentage difference between treated and control to evaluate the percentage of reduction (Reduction %), which is calculated with the following formula:

$$Reduction (\%) = \frac{(O_2 \cdot A_1) - (O_1 \cdot A_2)}{(O_2 \cdot P_1) - (O_1 \cdot P_2)} \times 100 \quad (2.8)$$

where O_1 is the molar extinction coefficient (E) of oxidized AB at 570 nm; O_2 is the E of oxidized AB at 600 nm; A_1 is the absorbance of test wells at 570 nm; A_2 is the absorbance of test wells at 600 nm; P_1 is the absorbance of control well at 570 nm; and P_2 is the absorbance of control well at 600 nm. The percentage of reduction for each sample was normalized to the percentage of reduction for the control (i.e. mCOLLs at day1 cultured in α -MEM) to obtain the cell viability percentage

For morphology assay, samples at day 10 were washed two times with PBS and fixed with 10% formaldehyde for 1h at 4°C. The fixed cells were permeabilized with Triton X-100 0.1% in PBS for 3–5 min. After 3 washes in PBS, the actin filaments of cytoskeleton were stained with 1:200 diluted FITC phalloidin (Cayman Chemical Company) in PBS for 30 min at room temperature. Finally, after three washes with PBS to remove unbound phalloidin conjugate, cell nuclei were stained with 1:10000 diluted 4,6-diamidino-2-phenylindole, DAPI, (SIGMA-ALDRICH) for 10 minutes at 37 °C. The samples were, then, observed by confocal microscope system (Leica TCS SP8) with a 40X and 63X oil immersion objective. Images were acquired with a resolution of 1024×1024 pixel. Eventually, microspheres morphology and 3D

distribution of hUCMSCs in mCOLLs was evaluated by using a specific analysis tool of LAS X (Life Science Microscope Software). In case of z-stacking, the thickness of each z-slice was set equal to 2 μm for cell morphology and equal to 10 μm when evaluating 3D distribution.

2.2.6 ATII expression study

In order to evaluate the differentiation capability of hUCMSCs into pulmonary ATII cells, primarily the immunofluorescence against Surfactant Protein C (SPC) antibody has been performed. Briefly, mCOLLs were cultured for 21 days in α -MEM, SAGM and SAGM+LMWHA. Then, they were fixed in 10% Formalin for 3h, permeabilized with 0.1% Triton X-100, blocked with 1% BSA and incubated with SPC rabbit antibody (Abcam) diluted in 1% BSA at 4°C overnight. After washing with PBS three times, FITC-conjugated secondary anti-rabbit antibody (Millipore, Billerica, MA, U.S.A.) was added to mCOLLs for 3 h at room temperature. In the end, cell nuclei were stained with blue DAPI for 10 minutes at 37°C. Samples were observed by confocal microscope system (Leica TCS SP5 MP) with a 63X oil immersion objective. Images were acquired with a resolution of 1024x1024 pixel. the thickness of each z-slice was set equal to 6.8 μm . To quantify the expression of the SP- C, the supernatants of mCOLLs culturing in α -MEM, SAGM and SAGM+LMWHA were collected for analysis after 21 days of exposure to the biomaterials, and then analysed using SP-C ELISA kit according to manufacturer protocol.

2.2.7 Statistical analysis

The results were expressed as mean $\pm$ standard deviation (SD). Data analysis was performed using Graphpad® software. The repeated results were compared with the one-way analysis of variance (ANOVA) and a $p - value < 0.0001$ was considered significant.

2.3 Results and discussion

2.3.1 HA solution rheological characterization

Rheological tests were performed to characterize the regime concentrations of different MW HA in solution, to have a more rational approach to the experiment design and data interpretation in the study. As a matter of fact, polymers in solution have different concentration regimes depending on their MW, and this can influence the interaction of another component in solution in terms of chemical physical and biological properties [33].

Flow curves were measured on LMWHA (**Figure 2.2A**), MMWHA (**Figure 2.2C**) and HMWHA (**Figure 2.2E**) at different concentrations, showing the typical shear-thinning behavior of polymeric solutions for all samples. The viscosity dependence of the shear rate was fitted with the Ellis fluid model (**Equation 2.1**) to obtain zero shear rate viscosities η_0 , used to calculate the specific viscosity η_{sp} (**Equation 2.2**).

The plots of η_{sp} against polymer concentration for LMWHA, MMWHA and HMWHA were reported in **Figure 2.2 B**, **Figure 2.2 D**, **Figure 2.2 F**, respectively. Three main linear trends with different slopes were detected, representative of a power-law dependency of η_{sp} from HA concentrations, in agreement with theoretical predictions. [34, 35]. The three linear trends, fitted with a straight line in the log-log plot, represent the three concentration regimes: dilute, semidilute unentangled and semidilute entangled regimes [36]. Slopes display such a concentration dependence of specific viscosity in agreement with theoretical predictions, to confirm that water for HA polymer is a good solvent [34, 35].

Data on overlap concentrations (C^*) and entanglement concentrations (C^e) were further plotted on a log-log graph as a function of molecular weight, to obtain a phase diagram for HA solutions. As a matter of fact, a linear arrangement of data can be noticed: performing a power law fit on data (translated to a linear relationship on bilogarithmic graph), dependencies from molecular weight of overlap and entanglement concentration can be respectively obtained as: $C^* = 4.5Mw^{-0.45}$ and $C^e = 115Mw^{-0.29}$. This enabled us to completely detect the three different dilution regimes for HA solutions (**Figure 2.3A**): dilute regime, semidilute unentangled regime and semidilute entangled regime.

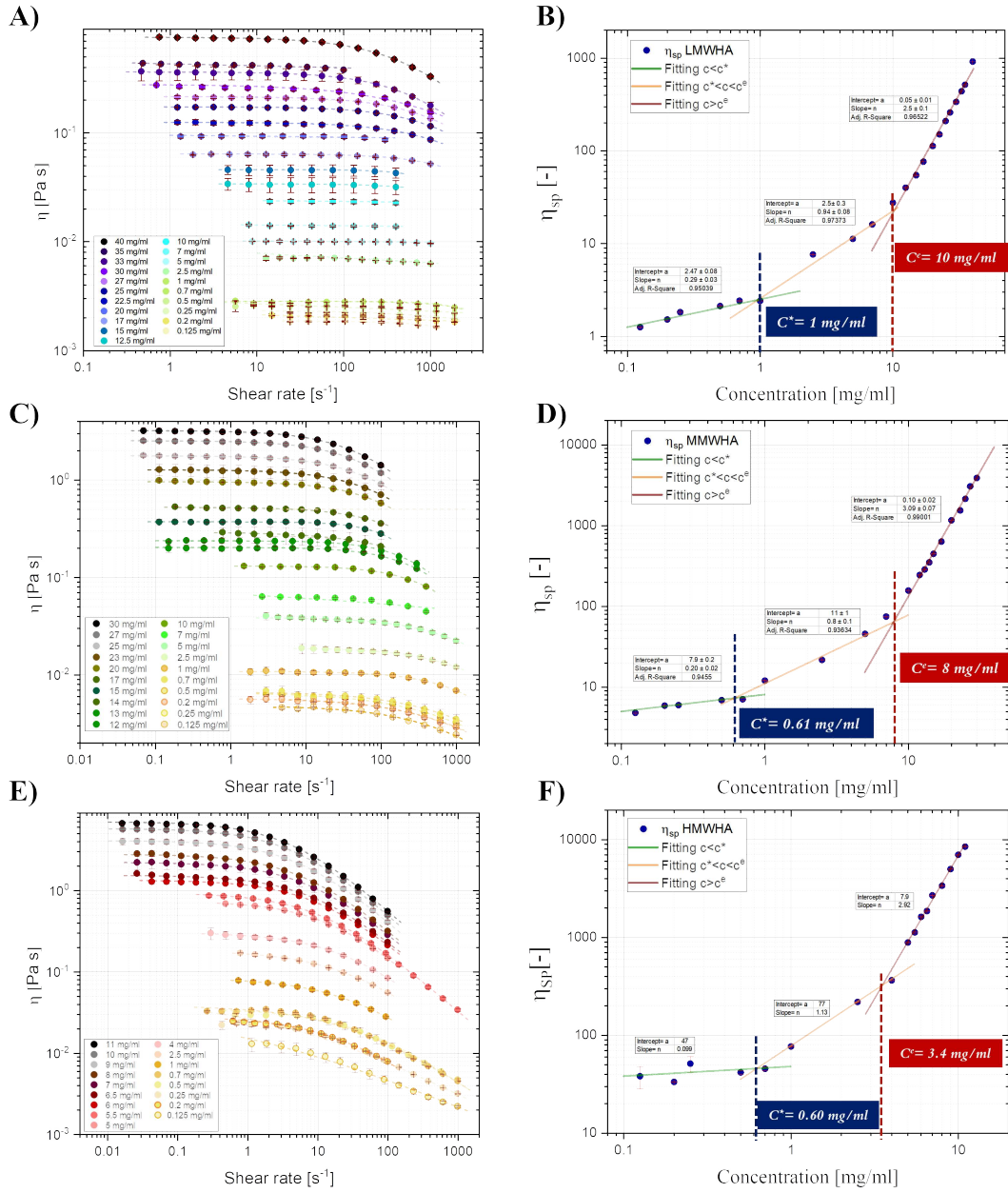


Figure 2.2: Rheological characterization of HA solutions. Flow curves of LMWHA (A), MMWHA (C) and HMWHA (E) at different concentrations; experimental triplicates were fit using the Ellis model (dashed lines). Zero shear viscosities obtained from the fit at different concentrations were used to plot specific viscosities as a function of HA concentration to obtain the critical overlap concentration C^* for the onset of semidilute unentangled solution and the critical entanglement concentration C^e for LMWHA (B), MMWHA (D) and HMWHA (F).

To further assess the reaching of an entangled regime, rheological oscillatory tests were performed, namely strain sweep (Figure 2.3B) and small amplitude frequency sweep (Figure 2.3C) on LMWHA, MMWHA and HMWHA.

Results collectively showed the presence of physical entanglements. Strain sweep showed

how at the chosen frequency ($f=1\text{Hz}$) solutions' behavior was liquid-like, with loss modulus G'' greater than storage modulus G' in all the strain range. Moreover, it can be noticed how at increasing molecular weight the fall of the modules happens to deformations gradually smaller and smaller, with LMWHA having the broader linear viscoelastic regime range. However, the smallest deformation value feasible also to investigate HMWHA solution, was used to conduct Frequency sweeps. It was, in fact, possible to identify crossover points between G' and G'' moduli, marking the passage from a liquid-like ($G''>G'$) to a solid-like behavior ($G'>G''$) at higher excitation frequencies.

This change in behavior is notably due to formation of entanglements that hinder the statistical movements of polysaccharidic chains. However, LMWHA solution show the lowest tendency to create entanglements between all molecular weights: even though the tested concentration is clearly gradually decreasing for LMWHA, MMWHA and HMWHA, the small scattering in G' and G'' points, enable us to infer that the smaller chain size distribution of LMWHA need a polymer concentration far beyond the C_e to sensibly assess the formation of entanglements. This problem does not occur with other molecular weights, for which, instead the crossover is clear. Besides, the crossover points shift at lower frequencies, suggesting that higher molecular solutions need more time to flow under an applied shear (i.e. higher relaxation times).

2.3.2 MSCs stemness phenotype evaluation by flow citometry

2.3.3 mCOLLs diameter characterization

Characterization of mCOLL diameter over time was performed to understand whether the systems could be suitable for pulmonary delivery. Representative images of mCOLLs diameter measurements have been shown in **Figure 2.4** at different times for different seeding (i.e. 1×10^5 and 1×10^6 cell/ml in α -MEM and 1×10^6 cell/ml in α -MEM/HA). Boxplots in **Figure 2.5A, B** and **C** represent the statistical dimensional distribution of the droplets in time. As a matter of fact, using pipetting as a technique to produce mCOLLs, allows to obtain droplets having an initial diameter between 1200 and 1600 μm , as it can be seen in boxplots (day 0). However, the ability of MSCs to contract collagen matrix microspheres significantly

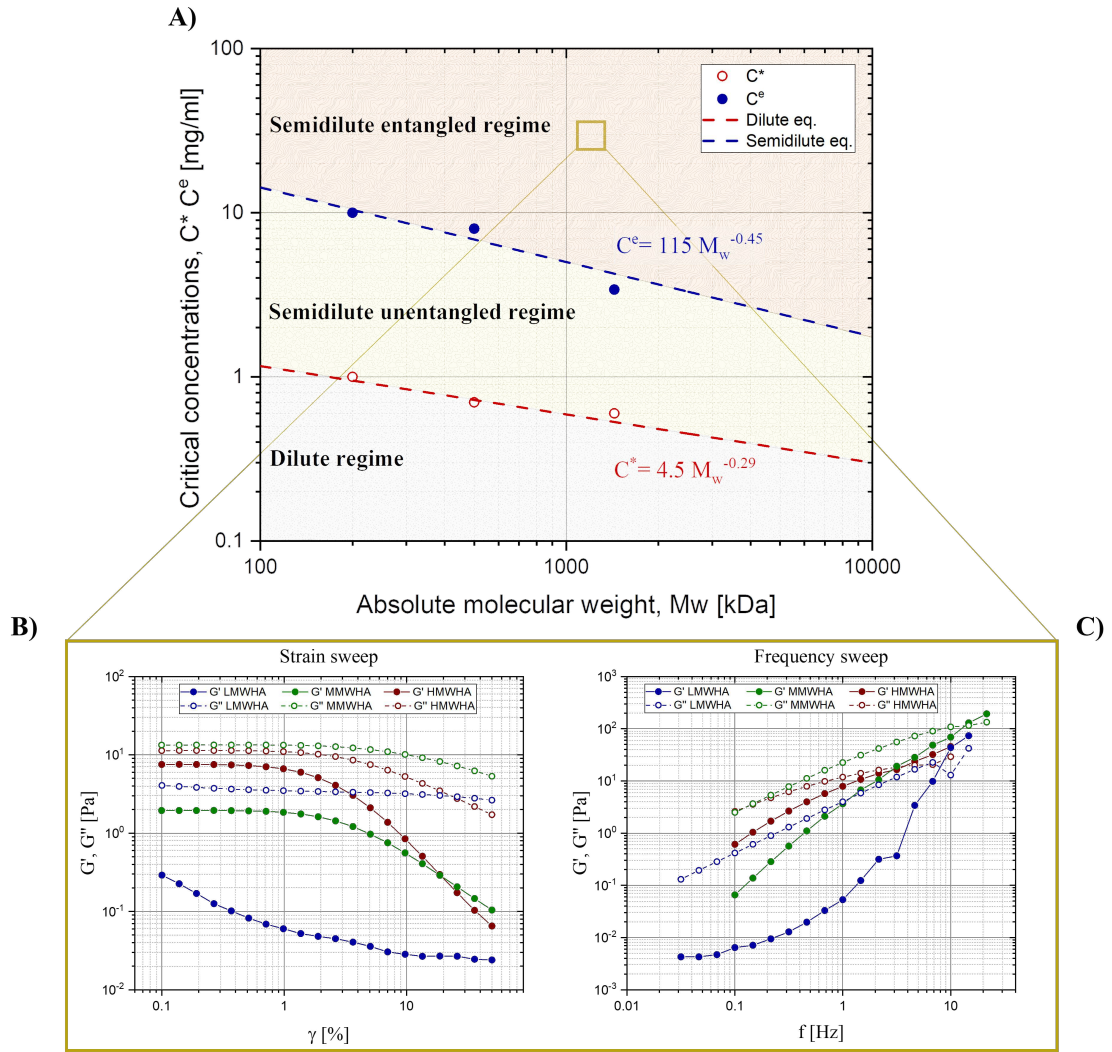


Figure 2.3: Rheological characterization of HA solutions. Data on critical concentrations for different HA molecular weights allow the identification of HA solutions phase diagram: diluted, semidilute unentangled and semidilute entangled regimes are highlighted and dashed lines represent the phase equilibrium lines (A). Representative oscillatory strain sweeps on LMWHA, MMWHA and HMWHA solution in the semidilute entangled regimes; Storage modulus (G') and loss modulus (G'') are reported as a function of deformation (frequency = 1 Hz, $T=25^\circ$) (B). Deformations for which linear viscoelastic region holds has been selected to run oscillatory frequency sweeps on LMWHA, MMWHA and HMWHA solution in the semidilute entangled regimes; Storage modulus (G') and loss modulus (G'') are reported as a function of oscillation frequency ($T=25^\circ$) (C).

decreased the average diameter of mCOLLS.

In particular, it can be seen how mCOLLS diameter tends to stabilize after a specific time. However, mCOLLS seeded at a density of 1×10^5 cell/ml in α -MEM showed, according to literature [37], a diameter, once stabilized, that was higher than the one of mCOLLS seeded at a density of 1×10^6 cell/ml. In order to study the effect of contractility alone on the dimension of the mCOLL, eliminating the variable of cell proliferation, the mCOLLS were seeded with MSCs at a density of 1×10^5 and 1×10^6 cell/ml treated with MMC (Figure 2.5 D). Indeed,

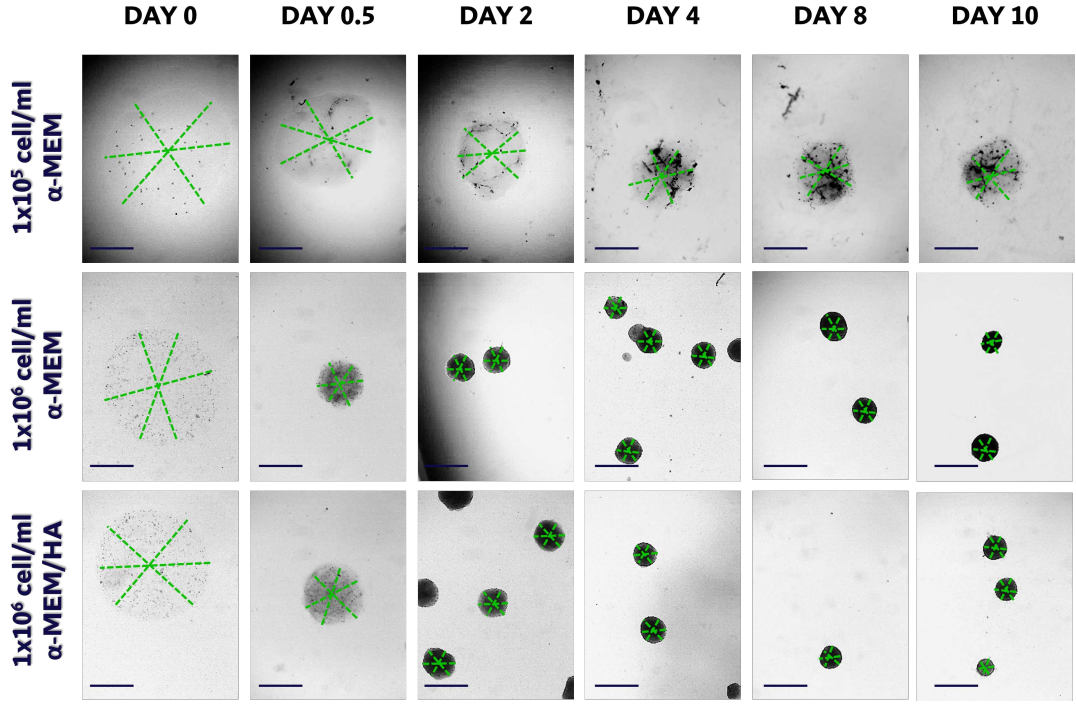


Figure 2.4: Representative images of mCOLLs diameter measurements at different times. Starting from the top, mCOLLs were seeded with 1×10^5 cells/mL in α -MEM, 1×10^6 cells/mL in α -MEM, and 1×10^6 cells/mL in α -MEM/HA as a biphasic delivery system. Red dashed lines represent all three different measurements of the mCOLLs diameter considered for statistical analysis. Scale bars represent $500 \mu\text{m}$.

MMC, which is commonly used in the protocol to promote differentiation as a feeder layer [32], interferes with DNA synthesis, blocking only cell proliferation, avoiding the apoptosis process, so vital cells can be seeded within mCOLLs [38]. Raw data has shown a dimension of $500 \pm 50 \mu\text{m}$ for the mCOLLs with MSCs-MMC treated seeded at 1×10^5 cells/mL while the dimension of $360 \pm 30 \mu\text{m}$ has been found for the MSCs-MMC treated seeded at 1×10^6 cells/mL (data not shown). Indeed, **Figure 2.5 D** shows the diameters (D) for mCOLLs seeded with MSCs at a density of 1×10^5 and 1×10^6 cell/mL treated with MMC normalised with respect to their individual diameters at day 0 ($D_{\text{day}0}$). The normalisation enables us to not consider the operator error in mCOLLs during pipetting. Besides, by hindering proliferation, it is safe to assume that all the cells acting on collagen contraction are the ones originally seeded. Thus, the general results observed indicated that mCOLLs treated with MMC reach larger diameters since fewer cells remain within the matrix and their contribution to contraction is clearly lower.

Furthermore, mCOLLs treated with MMC and seeded with 1×10^5 cells/mL achieved a smaller final diameter than those seeded with a lower number of cells. This suggested that the contribution of proliferation is a fundamental parameter to be taken into consideration for the

design of these systems once the appropriate cell density has been set so that the desired size of mCOLLs can be achieved. Once recognised that a seeding density of 1×10^5 cell/ml was not able to provide a sufficient diameter reduction to safely deliver mCOLLs through intratracheal administration, the further investigations were conducted focusing only on m COLL seeding density of 1×10^6 cell/ml.

Besides, the standard deviations deriving from the measurements of the three diameter for each mCOLL and for each day of culture was taken into account as a shape homogeneity parameter and was plotted against the culturing time in terms of $\sigma\sigma_0$ (with σ_0 being the standard deviation at day 0 for each sample) for mCOLLs seeded at a density of 1×10^6 cell/ml, in α -MEM and α -MEM/HA. Results showed how morphology of mCOLLs depends on the culture medium, since it reached smaller σ values for the h systems based on mCOLLs cultured in α -MEM/HA, indicating that they reach a more uniform shape distribution (**Figure 2.5E**). Indeed, mCOLL size of $194 \pm 50\mu m$ in α -MEM and of $156 \pm 40\mu m$ in α -MEM/HA have been obtained, suitable for injection, considering the bore size of 22 G syringe needle was around $413\mu m$, and considering that the site of interest of the realise in the alveolar sacs are about $400\mu m$ [39].

In order to describe a model for the variation of mCOLLs normalized diameter (D/D_0) with respect to days of culture, an empirical model of a plateauing decreasing exponential was used (**Figure 2.6**). The nonlinear fit gave as an output two values for each sample: the terminal reduced diameter D_∞ and a characteristic time k . The outputs showed how the fit was able to describe a lower terminal diameter for α -MEM/HA sample, with respect to α -MEM (being $D_\infty^{\alpha-MEM} = 271\mu m$ and $D_\infty^{\alpha-MEM/HA} = 260\mu m$) and a quicker stabilization of diameters for mCOLLs cultured in α -MEM/HA (being $k^{\alpha-MEM} = 2.8 days^{-1} = 3.24 \times 10^{-5} s^{-1}$ and $k^{\alpha-MEM/HA} = 1.6 days^{-1} = 1.85 \times 10^{-5} s^{-1}$).

The goodness of the chosen model can be deduced from the plot of standardized residuals d against the independent variable x (as reported in the top right corner of **Figure 2.6**. Their random distribution around the line $d = 0$, the fact that they are all enclosed in the range $-2 < d < +2$ and the fact that the width of the band in which the points fall varies with the experimental condition, allow us to infer the correctness of the model and that no systematic errors in measurements occurred. Experimental measurements are independent and random

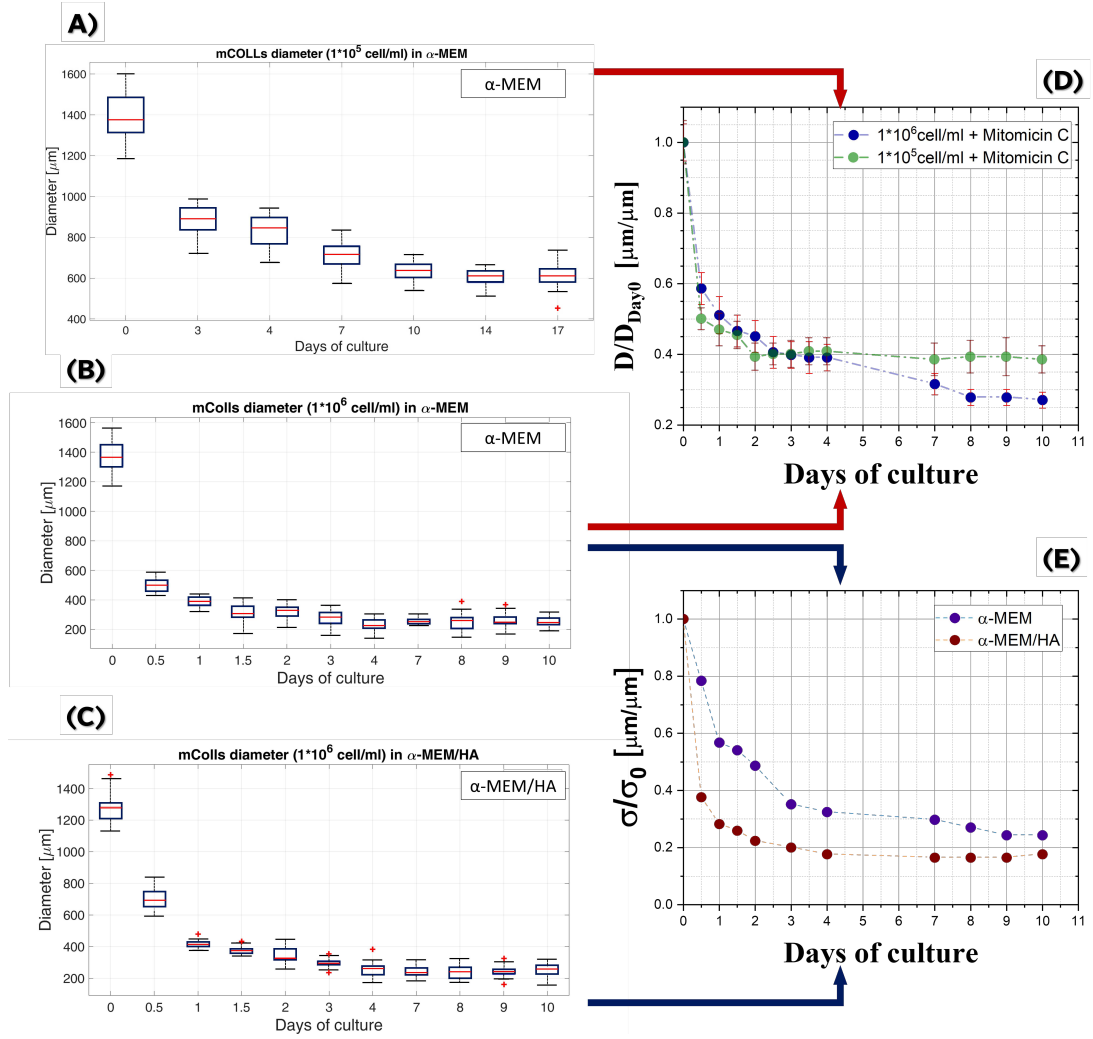


Figure 2.5: Dimensional characterization of mCOLLs. Boxplots representing statistical data analysis of mCOLLs diameter with respect to days of culture are reported in (A)-(C), highlighting the mean of measurements (red line), the first (q1) and second (q2) quartiles and the interquartile range (IQR) and possible outliers (red +). mCOLLs were seeded at 1×10^5 cell/ml in α -MEM (A), 1×10^6 cell/ml in α -MEM (B) and 1×10^6 cell/ml in α -MEM/HA as biphasic system (C). Effects of initial cell seeding density on mCOLLs contraction: normalized diameters measurements for MMC-treated mCOLLs are reported against the days of culture and confronted for mCOLLs seeded with 1×10^5 and 1×10^6 cell/ml, (D). Effects of culture medium on mCOLLs shape homogeneity: normalized standard deviations deriving from diameters measurements are reported against the days of culture and confronted for mCOLLs in α -MEM and in α -MEM/HA (E).

errors due to measurements are distributed in a Gaussian shape with a mean equal to zero and a variance not dependent on the independent variable (i.e. homoscedasticity) [40].

As a matter of fact, both data were subjected to a White Test for heteroscedasticity, and it was possible to conclude that none of the data suffered from heteroscedasticity. In particular, heteroscedasticity is a phenomenon that takes place when the residuals of a predicted variable, plotted in terms of an independent variable are non-constant, and so it can impact the validity of the tested model [41]. All these considerations can be derived from the value assumed by

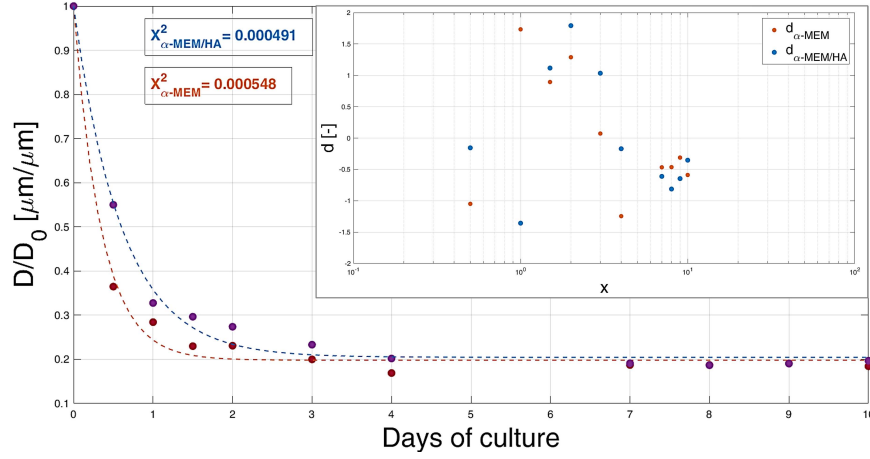


Figure 2.6: Non-linear curve fitting of normalized diameters (D/D_0) with respect to days of culture for mCOLLs in α -MEM and α -MEM/HA and a detail of standardized residuals distributions for the samples. Output of the non-linear fit are $D_{\infty}^{\alpha-MEM} = 271\mu m$; $D_{\infty}^{\alpha-MEM/HA} = 260\mu m$; $k^{\alpha-MEM} = 2.8 days^{-1}$; $k^{\alpha-MEM/HA} = 1.6 days^{-1}$.

the reduced χ^2 parameter, which is extremely low, indicating for both data populations a correlation in agreement with the proposed model.

2.3.4 Rheological characterization

In order to evaluate the potential injectability of the suspension of the biphasic system based on mCOLLs and HA solution for intratracheal administration, rheological characterization of the system was performed. Flow curves of different systems (including as control a suspension of PBS and MSCs at a density of 1×10^6 cell/ml, according to literature [7] are reported in **Figure 2.7 A**. Since nowadays standard clinical practice suggests the administration of PBS and MSCs first by aspiration in a syringe through a 22-G needle and then the injection through a 5-French feeding tube, imposing particularly high shear rates, we focused on this parameter. Results showed a shear thinning-like property of mCOLLs suspension in both PBS and PBS/HA, losing the Newtonian behavior of PBS alone. This is likely because mCOLLs, just as smaller-scale MSCs do, tend to line up when subjected to a high shear rate, reducing the stress needed to put in motion the fluid and therefore decreasing its viscosity.

Flow curves were fitted using the Cross model (**Equation 2.7**), and the terminal viscosities and their respective uncertainties were obtained as an output, reported in **Table 2.1**. PBS/HA + mCOLLs and PBS + mCOLLs or PBS/HA + MSCs and PBS+MSCs reach a terminal viscosity which is comparable to PBS/HA or PBS respectively. Then the relative ter-

terminal viscosities for all suspensions (η_∞) were reported normalised to control PBS+cell (η_∞^0), corresponding to the clinical practice [7].

Figure 2.7 B shows how the presence of HA in solution (PBS/HA and PBS/HA + MSCs) increases 4 times the ratio $\eta_\infty/\eta_\infty^0$ compared to PBS, PBS+cell and PBS+mCOLLs. Moreover, the increase was greater, about 5 times for the PBS/HA + mCOLL biphasic delivery systems. Overall, the results of the viscosity test demonstrated that all the suspensions analysed, despite the increase in values in the presence of HA, have a viscosity between $1 \times 10^{-3} Pa \cdot s$ and $1 \times 10^{-2} Pa \cdot s$. As reported in the literature, these viscosity ranges obtained are suitable for administration routes such as intratracheal [42, 43].

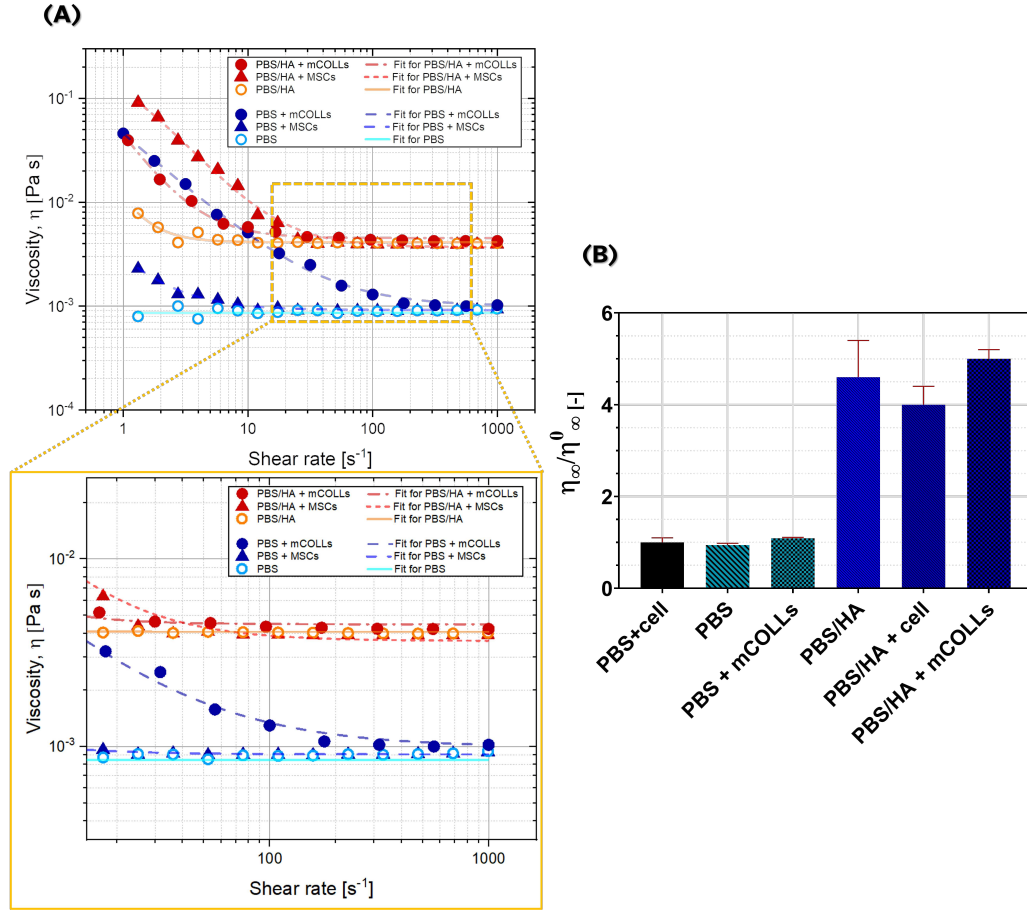


Figure 2.7: Rheological characterization of mCOLLs suspension in different media. **(A)** Flow curves of biphasic systems based on mCOLLs in PBS/HA (in red) and PBS (in blue) confronted with MSCs suspended in PBS/HA (rheological control) and PBS (state of art). Moreover, flow curves of solutions alone are reported (i.e. PBS in cyan and PBS/HA in orange). Error bars were omitted for the sake of clarity. **(B)** Terminal viscosities η_∞ compared to the state of the art (i.e. PBS+cell, η_∞^0). The terminal viscosity of PBS/HA+mCOLLs is about five times the CTR system.

Table 2.1: Comparison between terminal viscosities of samples. Results show a comparable rheological behavior between all samples, regarded as a mechanical control.

Sample	Terminal viscosity, η_{∞} [Pa s]
PBS	$(0.85 \pm 0.04) \cdot 10^{-3}$
PBS + cell	$(0.90 \pm 0.01) \cdot 10^{-3}$
PBS/HA	$(0.41 \pm 0.07) \cdot 10^{-2}$
PBS/HA + cell	$(0.36 \pm 0.04) \cdot 10^{-2}$
PBS/HA + mCOLLs	$(0.45 \pm 0.02) \cdot 10^{-2}$

2.3.5 Cell delivery of MSCs from mCOLLs

Once investigated by viscosity characterization, the practicability of the routes of administration, e.g. intratracheal, of these biphasic delivery systems, the potential of mCOLLs as cell delivery system has been tested. Therefore, the viability of MSCs embedded in mCOLLs was studied (**Figure 2.8 A**), and the expelled cells were monitored and counted as a function of days of culture (**Figure 2.8 B**). It can be seen how averagely HA enhances MSCs viability by penetrating inside the collagen matrix of mCOLLs. HA is widely known to enhance cell viability 44, and in this case the effect can be seen acting at least after 5 days of culturing, where a significance in viability can be seen with respect to mCOLLs cultured in α -MEM for the same time.

Such significance is preserved for the following days as well: α -MEM/HA sample shows high significance with respect to α -MEM especially after 9 days of culturing. However, a pattern can be observed, as viabilities seem to lower after 6 days of culture, probably due to the fact that cells are being expelled from mCOLLs and the reduction percentage of Alamar Blue in mCOLLs lowers. Then a slight increase in viabilities is observed after 9 and 12 days; this behavior might be mainly due to the continuous proliferation of MSCs inside the mCOLLs, acting in synchrony with the phenomenon of expulsion.

In order to assess the hypothesis of cell expulsion, pivotal in a cell delivery system, cell counting of expelled cells monitored during the culturing of mCOLLs in α -MEM/HA has been performed. It can be seen how the release of cells from mCOLLs, initially seeded with 1×10^6 cells, is time-dependent. Indeed, after one day of incubation mCOLLs release about 14.000 MSCs. This can be due to the assessment of the system, since in this phase probably, the contraction phenomena (such as the cell-cell and cell-collagen interactions) and the prolifera-

tion phenomena have not yet reached saturation, in accordance with the previous results on the diameter characterization (**Figure 2.4**) and the following morphological characterization (**Figure 6**).

The number of expelled cells quickly decreases even after another day of incubation (i.e. day 2), as slightly less than 5.000 cells are expelled. Eventually, when proliferation inside the mCOLLs occurs in parallel with expulsion from the microdroplets, an equilibrium is reached in terms of expelled cells, as an average of 1000 cells are expelled day by day. This sustained release of MSCs is a desirable property of mCOLLs, since the aim of such substrates is to behave as a biomimetic niche and delivery platform for MSCs, once their diameter is optimized for the specific route of administration (i.e. intratracheal) and once the damaged pulmonary site has been reached.

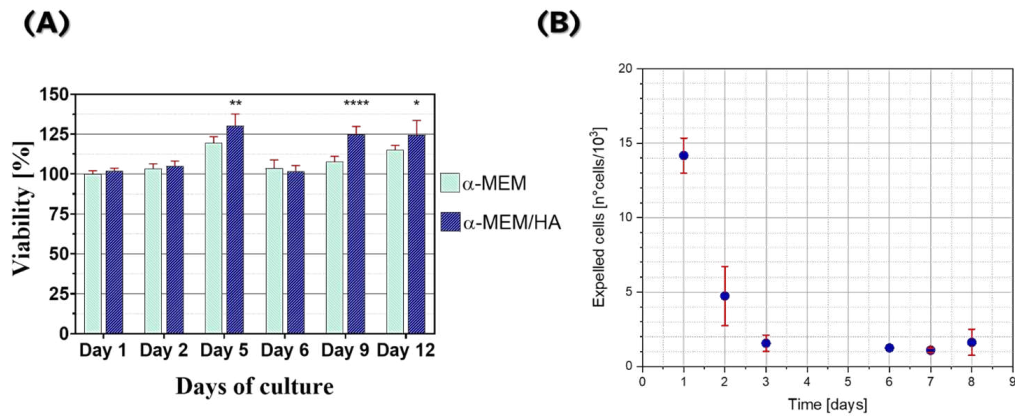


Figure 2.8: (A) Viability of mCOLLs cultured in α -MEM and α -MEM implemented with LMWHA as biphasic delivery systems at 1,2,5,6,9,12 days; **** p <0.0001 vs. Day 9 α -MEM, ** p <0.01 vs. Day 5 α -MEM, * p <0.05 vs. Day 12 α -MEM. (B) Number of expelled MSCs from mCOLLs cultured in α -MEM/HA starting from 1×10^6 cells. Data on y-axis were divided by 10^3 for the sake of clarity.

2.4 Conclusions

These results place a fundamental piece in the knowledge of polymeric substrates that can be used in the stem cell therapy strategies in lung regenerative medicine. In particular, HA macromolecules promoted the differentiation of MSCs compared the normal medium used to induce *in vitro* pulmonary differentiation indicating that HA could be further explored for the repair of alveolus damage and in lung regenerative medicine.

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Chapter 3

Folic Acid-based carbon dots: synthesis optimization for enhanced antimicrobial activity against lung-related pathogens

Contents

3.1 Introduction	72
3.2 Materials and methods	75
3.2.1 Microwave-assisted synthesis, purification and yield	75
3.2.2 Zeta potential measurements	76
3.2.3 Fourier-Transformed Infrared (FT-IR)	77
3.2.4 Thermogravimetric analysis (TGA) of precursors and FA-CDs	77
3.2.5 UV-Vis absorbance	77
3.2.6 Biological properties	78
3.2.7 Antimicrobial activity	79
3.3 Results and discussion	80
3.3.1 Microwave assisted CDs synthesis from FA and urea	80

3.3.2	Zeta potential measurement	81
3.3.3	Fourier-Transformed Infrared (FT-IR)	87
3.3.4	Thermogravimetric and Differential Thermogravimetric analysis (TGA and DTG) of precursors and CDs	88
3.3.5	UV-vis absorbances	91
3.3.6	NHLFs cells viability assay and morphological analysis	92
3.3.7	Antimicrobial activity and oxidase-like activity by monitoring ROS generation	95
3.4	Conclusions and future perspectives	98

3.1 Introduction

Even though respiratory system is physiologically populated by several bacteria, some bacterial infections pose a significant challenge in the realm of respiratory diseases, especially when facing COPD and acute respiratory distress syndrome (ARDS) [1]. As a matter of fact, microbiota has been seen changing when contracting COPD and these infections can also exacerbate the underlying conditions, leading to heightened morbidity and mortality rates [2].

COPD represents a chronic pathology, progressive and partially reversible airflow obstruction, with lung hyperinflation and significant extra pulmonary manifestations, mainly caused by long-term exposure to harmful gases or particles [3]. Here, bacterial infections can play a pivotal role in COPD exacerbations, leading to accelerating impairing of lung functions. It has been reported that acute exacerbation of COPD was responsible for 1.5 million emergency department visits, 726,000 hospitalizations, and approximately 119,000 deaths in 2000 [4, 5]. In particular, among the pathogens frequently implicated in COPD exacerbations are *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and more [6, 7].

Conversely, ARDS has proved to be one of the major clinical problems in respiratory medicine. Even though its definition is everchanging due to different and contrasting outcome data, it is characterized by sudden and severe respiratory failure, often precipitated by various underlying conditions such as pneumonia, sepsis, or trauma [8, 9]. In the context of ARDS, bacterial infections commonly involve pathogens like *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* [10]. These infections further compromise lung function, exacerbating the already precarious respiratory status of affected individuals.

Addressing the problem of bacterial infections in ARDS and COPD necessitates a multifaceted approach, especially in terms of an appropriate antimicrobial therapy. This antimicrobial therapy seems to be of pivotal importance, especially due to the increasing problem of multidrug resistance by microorganisms, mainly due to overconsumption of antibiotics, as well as their broad utilization in agriculture and also due to the non-availability of novel antibiotics [11].

In order to overcome multidrug resistance effects, the use of nanostructured systems has been spreading lately, thanks to their ease in scalability, low cost, and versatility [12]. Moreover, also other advantages related to higher biocompatibility than traditional antibiotics, as well as longer duration [13].

Therefore, by comprehensively understanding the complexities and tunability of antibacterial properties of nanomaterials in acute and chronic lung diseases, it is possible to improve outcomes and reduce the burden of respiratory morbidity and mortality. Notably nanoparticles (NPs) have been widely used in numerous medical applications, especially biosensing, drug delivery, bioimaging, and antimicrobial therapy. However, a specific category of nanoparticle, carbon quantum dots (CDs) are a promising class of carbon-based photoluminescent nanomaterials with several properties, such as biocompatibility, low toxicity, low cost and ease in functionalization [14, 15]. More importantly they have been shown to have intrinsic broad-spectrum antibacterial activity.

CDs have already been used in cancer therapy, but have also shown to have the property of producing reactive oxygen species (ROS), which are known to be able to kill bacteria [16]. However it has been seen a wide variety of effect of CDs on bacteria, since they can act by different mechanism of action, also according to their particular chemistry, size, doping and functionalization [17]. Those mechanism includes the adhesion of CDs to the bacterial surface, resulting not only in the isolation of bacteria from the growth medium, but also in the leakage of some intracellular components. Also it was reported the capability of CDs of binding to bacteria DNA [18].

We choose folic acid (FA) as main precursor of CDs, since it allows the formation of strongly N-doped CDs, thanks to FA chemistry. The chemical structure of FA is quite complex and presents several moieties. It contains a heterocyclic pterin structure with C and N atoms, having a methyl group in the sixth position bridging it to a para-aminobenzoic acid, which is further covalently linked to a L-glutamic acid (see structure in **Figure B.2**). It is found commonly in mammals, bacteria, algae, etc. and some of its main roles is intra-cellular signaling, ultraviolet protection, and fluorescent pigment [19].

Urea, on the other hand has been used to favor the synthesis, acting both as a catalyst

and a reagent able to add nitrogen group in the carbon-based graphitic structures in the CDs. In particular, urea is the is the diamide of carbonic acid, symmetrically composed of two amino groups (-NH₂) that are joined by a carbonyl group (-C(=O)-) and its role in metabolism of nitrogen-containing compounds is fundamental [20].

The presence of N-doping was actually seen to have a strong effect on antimicrobial properties [17]. Moreover it has been interestingly seen that FA-based CDs (FA-CDs) are able to respond to the environmental stimuli of bacterial infections, in particular pH, acquiring antimicrobial capacity “on demand” as especially acting on biofilm disruption [21]. Besides, it also enables to produce highly-luminescent CDs and is an excellent way to promote its efficient and specific target to folate receptor expressed cells [22].

This work wants to make use of a microwave-assisted and accurately monitored system to produce FA-CDs, strongly controlled in terms of size distribution, doping, chemistry and functionalization. The work aims at studying the influence that one of the main reaction parameters (i.e. reaction temperature) have on CDs physico-chemical properties and how such structural properties eventually affect CDs functional properties in terms of application against different types of bacteria.

In particular, the antimicrobial study will more thoroughly focus on bacteria proven to play a major role in lung-related pathologies, as well as notably showing a multi-drug resistance effect. To evaluate the application in lung-related pathologies, also bio-interaction with lung cells is studied.

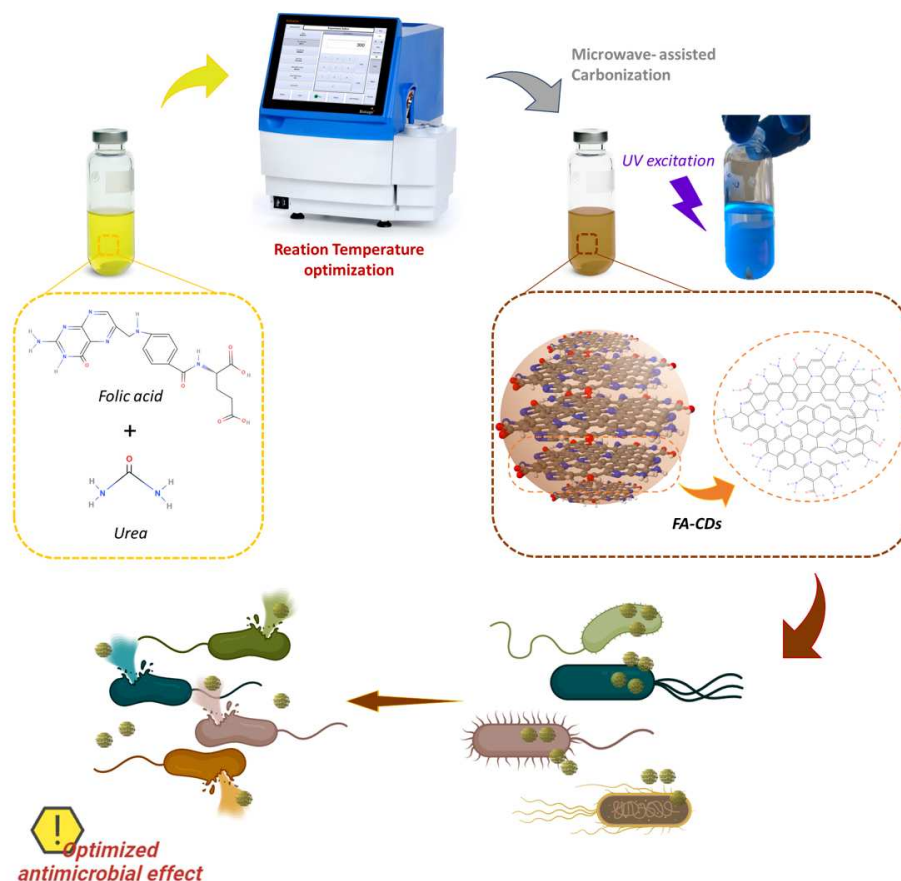


Figure 3.1: Scheme of the study. FA and urea were used as precursors to obtain a N-doped FA-based CDs by a highly controlled microwave-assisted method. In this study the reaction temperature was biased, in order to obtain photo-luminescent CDs, with optimized antimicrobial properties.

3.2 Materials and methods

3.2.1 Microwave-assisted synthesis, purification and yield

The FA-CDs were prepared using FA (Sigma) and urea (Sigma) as precursors by one-step microwave assisted method. Firstly, 200 mg of FA and 200 mg of urea were dissolved in 10 mL of bidistilled water (pH=7) and added in a 10-20ml glass reaction tube to form precursors solution. After 15 min ultrasonication at 59 Hz the reaction tube was put in *Biotage Initiator*⁺ microwave synthesizer for a fixed reaction time $t=10$ min, under different reaction temperatures ($T=130, 170$ and 185 °C, see **Table 3.1**) and a fixed magnetic stirring velocity of 600 rpm¹.

¹For the future, it could be interesting to test each reaction temperature also at pH =10, in order to evaluate the possible effect of pH on the yield and stability of the CDs due to the higher degree of solubilization of the

Once reacted, the system changed color, transitioning from a yellowish turbid to a brownish clear colloidal dispersion.

Table 3.1: Different synthesis protocols for different FA-CDs samples.

Sample	Reaction	Reaction time	Precursors solution
	temperature [°C]	[min]	pH
LT/CDs	130	10	pH=7 (water)
MT/CDs	170	10	pH=7 (water)
HT/CDs	185	10	pH=7 (water)

The suspension was preliminarily centrifuged at 13.000 rpm for 15 minutes, in order to get rid of eventual carbonization residues. The supernatant was withdrawn and further dialysed for one day in alkaline water (pH~9), using dialysis membranes having a 0.5-1 kDa MWCO, in order to dispose of possible unreacted precursors, as well as low molecular weight reaction residues. The dialyzed product was, then, freeze dried for 2 to 3 days, strongly depending on the amount of water in the samples, since the CDs are able to bind water molecules through H bonding.

3.2.2 Zeta potential measurements

Zeta Potential (ζ) measurements were performed using a Zetasizer Nano (Malvern Instruments, Malvern, UK) in order to evaluate the effect of different synthesis protocols on FA-CDs stability.

Dust-free solutions were obtained by filtration through cellulose acetate membrane filters with a pore size of (0.45 μm). The concentration of FA-CDs dispersions was 5 mg/ml at a set temperature of 25 °C. Then, one sample (HT/CDs) was chosen as a model to evaluate the temperature dependence on a higher pH range, this time from. To convert electrophoretic mobility was converted into ζ potential the Smoluchowski approximation was assumed.

precursors and stability of obtained CDs.

3.2.3 Fourier-Transformed Infrared (FT-IR)

To the aim of preliminarily investigate the formation of CDs in terms of rearrangement of molecular bonds, FA-CDs obtained from different reaction protocols were characterized using Perkin Elmer Frontier Fourier Transform Infrared Spectroscopy FT-IR (Waltham, MA, USA), with a single-reflection, universal ATR-IR accessory. All spectra were recorded between 4000 and 650 cm^{-1} with a resolution of 4cm^{-1} .

3.2.4 Thermogravimetric analysis (TGA) of precursors and FA-CDs

TGA was preliminarily performed on the precursors (i.e. FA and urea) to study their stability in the range of reaction temperatures investigated and then, also performed on the synthesized FA-CDs in order to highlight the important phases of weight loss. Experiments were conducted with Pt sample holders, using approximately 7 mg as mass of FA, urea and CDs.

A temperature ramp was set from 25 to 700 $^{\circ}\text{C}$, with a nominal heating rate of 4 K/min under nitrogen flow for precursors analysis. Instead, in order to deduce some eventual structural differences among differently synthesized FA-CDs, a TGA/DTG was also performed on CDs. Therefore, a temperature ramp was set from 40 to 1000 $^{\circ}\text{C}$, with a nominal heating rate of 10 K/min under nitrogen flow.

3.2.5 UV-Vis absorbance

Ultraviolet-visible (UV-vis) absorption spectra allow to draw conclusions about the effect of size distribution on quantum confinement effects. FA-CDs were dispersed in ultrapure water and to a concentration of $10\mu\text{g}/\text{ml}$ and UV-vis spectra were recorded using a PerkinElmer Lambda 900 spectrophotometer in a standard 10-mm quartz cuvette. Analysis was conducted in the 200-550 wavelength range, with 1nm spectral resolution, 1 nm/s acquisition rate and 2nm slit settings.

3.2.6 Biological properties

Cell Culture and viability assay

The NHLFs (Normal Human Lung Fibroblast, Primary) cells were grown in T-75 cell culture flask (Falcon, Italy) in complete Fibroblast Growth Kit-Low Serum, supplemented with rh FGF b, (0.5 mL, 5 ng/mL), L-glutamine (18.75 mL, 7.5 mM), Ascorbic acid (0.5 mL, 50 $\mu\text{g/ml}$), Hydrocortisone Hemisuccinate (0.5 mL, 1 $\mu\text{g/ml}$), rh Insulin (0.5 mL, 5 $\mu\text{g/ml}$), FBS (10.0 mL, 2%), in a humidified and controlled atmosphere at 37 °C and 5% CO₂.

The medium was changed every 3-4 days. The cells were detached, after confluent growth was reached, with 0.25% trypsin - EDTA solution and washed twice with PBS. The resulting cell suspensions were centrifuged (5 min, 1200 rpm; BRK55/10 Centrifuge by Centurion Scientific Ltd, UK), the supernatant separated, and the cells re-suspended in fresh culture medium. Viable cells were counted using the TC20 automated Cell Counter (Biorad, USA). In order to understand the *in vitro* biocompatibility of FA-CDs, their cytotoxicity was studied. To do so, cells were seeded at a density of 2×10^3 cells/ml on 96-wells (World Precision Instruments, Inc).

The cells were seeded for each well in triplicate cultured up to 24h. After 24 h, the culture media was replaced with 200 μL of medium containing different protocols CDs solution at pH 7 at a concentration (0, 50, 125, 250, 500 and 1000 $\mu\text{g/ml}$). Then Alamar blue assay (AB) was performed by adding AB reagent to the samples (at 10% v/v with respect to the culture medium) and incubated at 37 °C for 4 hours. The absorbance of the samples was measured using a spectrophotometer plate reader (Multilabel Counter, 1420 Victor, Perkin Elmer) at 570 nm and 600 nm.

Data are expressed as the percentage difference between treated and control to evaluate the percentage of reduction, which is calculated with **Equation 2.8**. The percentage of reduction for each sample was normalized to the percentage of reduction for the control to obtain the cell viability percentage. Experimental data were acquired for different incubation time (1, 3, 5 and 7 days of culture) with CDs.

NHLFs cells morphology

Fluorescence microscopy was further used to observe NHLFs morphology after the 7 days incubation with CDs. In order to remove deposited CDs, the samples were washed with PBS (1×), three times, fixed with 10% formalin (Sigma-Aldrich) for 3 h, and permeabilized with 0.1% Triton X-100 for 3–5 min. The actin filaments were stained with TRITC phalloidin for 30 min at 25 °C. Then, cell nuclei were stained with blue DAPI for 10 min at 37 °C, after two washes with PBS. Samples were observed by JuLITM Stage microscopy (Nanoentek) with a 20× objective. Images were acquired with a resolution of 1024 × 1024 pixels.

ROS generation by L-Ascorbic Acid absorbance reduction

To study the oxidase-like activity of FA-CDs in terms of ROS generation, the progressive disappearance of L-ascorbic acid (AA) maximum absorption peak at 266 nm after oxidation to dehydroascorbic acid, was monitored. Briefly, an AA working solution was prepared in PBS 1X (0.2 mM). Then, 100 μ l of AA solution were dissolved in PBS 1X as positive control, H_2O_2 10mM as a negative control and in LT, MT and HT FA-CDs (at 17.5 and 50 μ g/ml concentration) to a final volume of 2 mL.

The solutions were incubated at 37°C under gentle stirring for 4h, then UV-vis absorbance spectra were recorded using a PerkinElmer Lambda 900 spectrophotometer in a standard 10-mm quartz cuvette. Analysis was conducted in the 200-450 nm wavelength range, with 1nm spectral resolution, 1 nm/s acquisition rate and 1nm slit settings. Eventually, the absorption peak reduction for each sample was normalized to the positive control (i.e. PBS 1X) absorbance peak value to obtain an absorbance reduction percentage.

3.2.7 Antimicrobial activity

Overnight cultures of *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA) grown in Brain Heart Infusion Broth (BHI, Oxid) with an incubation temperature of 37 °C were used to prepare bacterial cell suspension for antibacterial activity tests. Bacterial cells were washed twice with saline solution and adjusted to a turbidity level equal to 0.5 McFarland

standard (BioLife, Italy).

The bacterial growth measurement was performed in 96-well plates. Each well contained 20 μ l of bacterial cell suspension and various concentrations (7500, 3750, 1875, 938, 469 and 234 μ g/ml) of CDs obtained by the different protocols in BHI medium. The analysis was performed in triplicate. The treated bacterial samples were then incubated at 35 °C for 24h. The optical densities (OD) of the samples were measured at wavelength of 600 nm after incubation using Multiskan FC Microplate Photometer (ThermoFisher Scientific). Inhibitory effect of treatment on bacterial growth was evaluated based on a decrease in the OD600 values compared to those for the untreated control samples. The percentage of bacterial growth was calculated by divided the OD of each samples for the OD of control untreated group.

3.3 Results and discussion

3.3.1 Microwave assisted CDs synthesis from FA and urea

BiotageInitiator⁺ microwave synthesizer was able to conduct a microwave synthesis of materials under pressurized conditions and precisely monitor reaction parameters (i.e. supplied power, temperature increase and pressure in reaction vessel). As a matter of fact this specific reaction system shows all the advantages of microwave-assisted synthesis (direct heating of target molecules, faster reaction times [23]) with some additional features overcoming the most common synthesis by domestic microwave irradiation: an excellent reproducibility, a higher yields and purer compounds with extremely precise and controllable size distribution [24].

In **Figure 3.2** reaction plots are depicted: correlating the power supplied by the synthesizer with the temperature read by the thermocouple in the reaction vessel, as well as the pressure in chamber, which is notably supposed to affect the reaction yield since chemical reactions are more likely to occur under high pressure [25]. In this reaction configuration even lower temperatures than conventional microwave assisted synthesis can be used, which may directly lower the risk of precursors degradation and -therefore- increase reaction yield.

The conversion of ammonium cyanate to gaseous ammonia in urea degradation path

can be one of the causes of the continuous increasing pressure of the system when T stabilizes for MT and HT samples. As a matter of fact, pressure averagely stabilizes when reacting at $T=130^{\circ}\text{C}$, but it does not reach the same plateau at higher temperatures, because the system is continuously generating ammonia, progressively increasing the pressure in chamber (apart from the contribute that increasing temperature brings to the pressure increase).

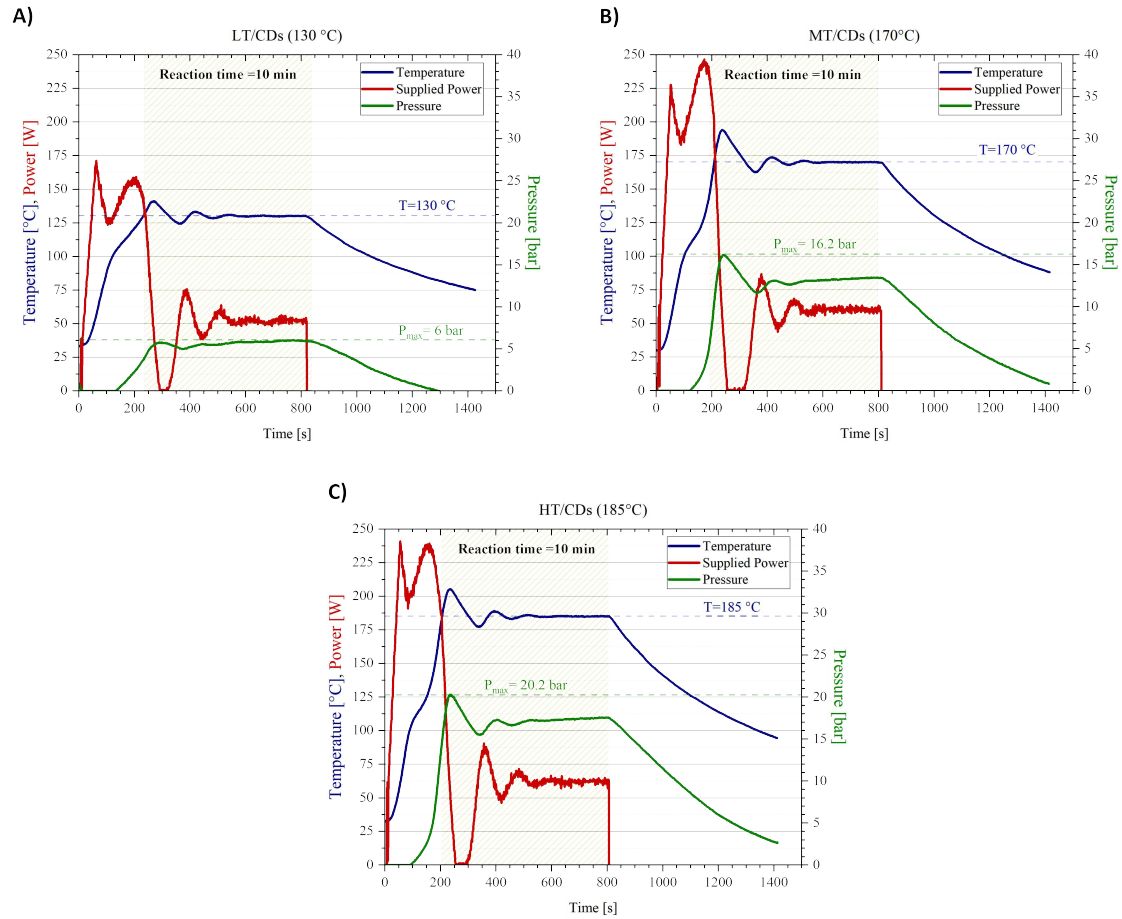


Figure 3.2: Synthesis protocols plots for CDs at different reaction temperatures. Temperature (blue), supplied power (red) and pressure (green) monitoring during reactions for CDs synthesized at 130 °C (A), 170 °C (B) and 170 °C (C).

3.3.2 Zeta potential measurement

The zeta potential of the different CDs was measured after they were suspended in different pH solutions. As a matter of fact, the presence of ions (e.g. H^+ or OH^- ions) has a strong effect on the surface charge of many types of particles².

²Even though the ζ potential is often qualitatively defined as an electrostatic potential at a non-well-defined slip plane near a solid surface, actually identifying its location is not possible. This means that is not correct to match the ζ potential value with a particular point on the electrostatic potential profile [26].

The different pHs were obtained starting from two solutions: the most alkaline and the most acidic one and then dilute them with Milli-Q water to the other pH values. This was done to avoid the acid-base neutralization which could have changed the ionic strength of the solution, interfering with the measurement³ [27]. The negative zeta potential exhibited at neutral pH showed how the doping of two electron-rich groups on the surface influences the surface charge [28]. In particular it is influenced by N-rich groups -such as $-NH_2$ - on the surface and abundant oxygen-containing groups, like $-OH$ and $-COOH$. The fact that the zeta values are roughly all in the same range within the limits of experimental uncertainty, is linked to the fact that all CDs share the common feature of hydroxyl and carbonyl groups heavily decorating the surface.

However, the difference in the isoelectric (IEP) point of the different CDs suspension seems to highlight a structural difference among the samples in terms of functional groups decorating the surface⁴ (**Figure 3.3**). As a matter of fact, the isoelectric point represents the pH value at which it can be seen how the pH value at which this change happens can be obtained by finding the point in which the linear fit of the different measurements crosses the $\zeta = 0\text{ mV}$ line (i.e. 4.7, 4.4 and 3.0 for LT/CDs, MT/CDS and HT/CDs respectively). The difference in the isoelectric points for the three samples is related to a different distribution of surface groups on CDs. Apparently, the most significant difference is seen with the HT/CDs sample, which shows a smaller isoelectric point. Now, since an incipiently stable suspension is that for which pH is one full pH unit away from the IEP, the HT/CDs showed a slightly better colloidal stability.

Now, since ζ potential measurements come from an electrophoretic migration measurement, this analysis can give also information on their stability in solution. Therefore, ζ potential was further investigated, increasing the pH range and testing one of the CDs samples (i.e. HT/CDs) at different temperatures (i.e. 4°C, 25°C and 37°C), in order to deepen the surface charge and the interactions with the solution and among the CDs themselves [29]. From this point of view the ζ potential was used as a tool to study the CDs' performance, also in terms of improving their dispersion when dealing with them at different study stages (**Figure 3.4**).

³As a matter of fact, the ζ potential depends not only on the properties of the solid surface itself, but also on the nature of the ions in solution.

⁴At pH values lower than the IEP, the particle surface charge is positive and at pH values higher than the IEP, the particle surface charge is negative.

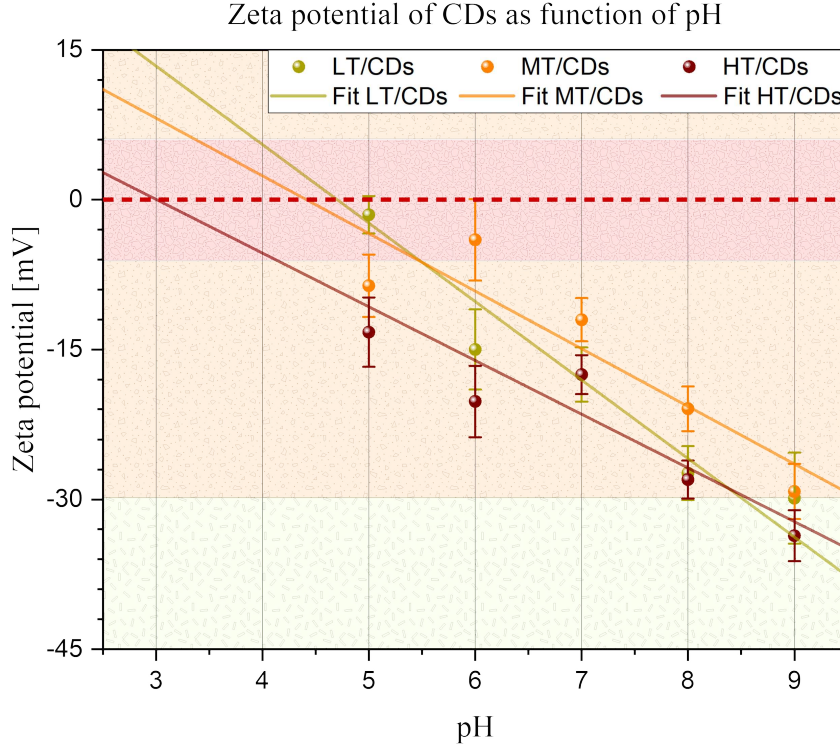


Figure 3.3: Zeta potential of CDs as a function of pH. Solid lines represent linear fits for all samples. The points in which fit lines cross the dashed **red line**, represent the isoelectric point for each sample (i.e. 4.7, 4.4 and 3.0 for LT/CDs, MT/CDS and HT/CDs respectively).

In particular, the surface potential was monitored at the three most significant temperatures: 4°C was taken as the storage temperature, 25°C as the average handling temperature in most of the passages and experiments, while 37°C is representing of their use as biomedical devices, since it corresponds to body temperature and is the actual temperature of the suspension during all biological assays.

It can be seen how the behavior seems to be mildly temperature-dependent, as the curve shape is overall preserved at the three different temperatures. In order to figure out what the actual dependence could be, an attempt has been done to fit all data points with a 2D planar fit (see **Appendix C**) and the result delivered the following outcome for the functional dependency:

$$\zeta(pH, T) = -5.81 pH + 0.04 T + 22.6 \quad (3.1)$$

This means that the dependence of zeta potential on temperature is negligible compared to the dependence on pH, as:

$$\left| \frac{\partial \zeta}{\partial pH} \right| = 5.81 \gg 0.04 = \left| \frac{\partial \zeta}{\partial T} \right| \quad (3.2)$$

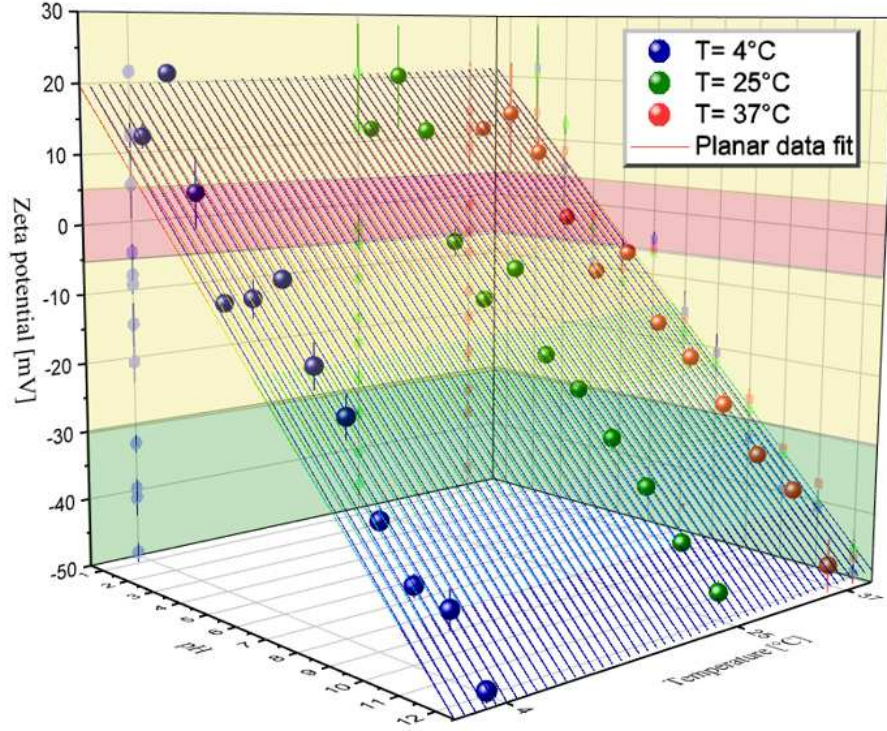


Figure 3.4: Model CDs (HT/CDs) ζ -potential dependence on pH and temperature by a three-dimensional plot over three temperatures (namely 4 °C, 25 °C and 37 °C). The data are fit with a 2D plane fit, represented by a multicolored plane in the 3D plot. On the two 2-D planes representing the ζ vs T and the ζ vs pH plots the 3D points projections are represented. On the 2-D graphs are then highlighted in **red** the *rapid agglomeration zone*, in **yellow** the *incipient stability zone* and in **green** the *good stability zone*. Error bars are represented as a solid vertical line on data points.

The strong dependence of ζ potential from pH can be better focused, highlighting the 2D plot deriving from the projection of the 3D data point on the $\zeta - pH$ plane, obtaining that way the plot on **Figure 3.5**. Again, there is an overlap of most of the dataset point at different temperatures.

the only difference can be grasped when evaluating the IEP by finding the crossover point of the linear fit of each dataset with the $\zeta = 0\text{ mV}$ line. It can be seen how it ranges from a pH value of 2.5 to 4, even though no clear temperature relation was found. However, extending the pH analysis range gave a better outlook to the effect that solution acidity has on the CDs stability.

As a matter of fact, it can be seen how pH values deeply influence the net surface charge, since the H^+ and the OH^- ions have an effect on protonation and deprotonation of chemical moieties on the external surface of CDs (namely $-COOH$, $-NH_2$ and $-OH$ groups), as shown

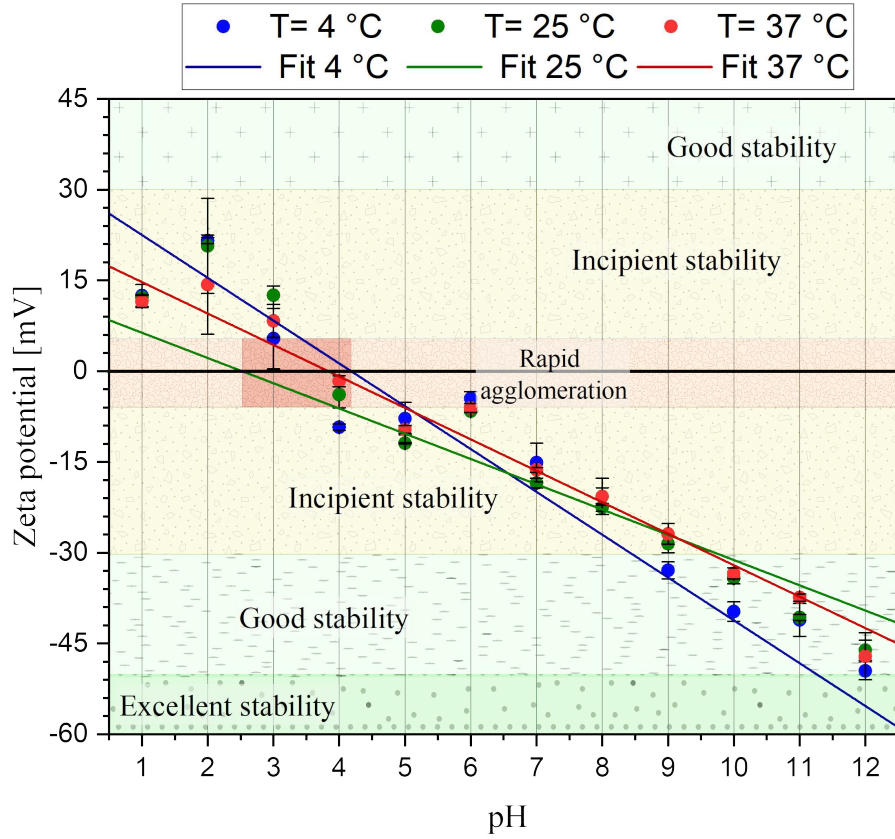


Figure 3.5: ζ vs pH 2D plot at different temperatures from the the 3D plot of Figure 3.4. Solid lines represent linear fits for all samples. The points in which fit lines cross the solid horizontal **black** line, represent the isoelectric point for the sample tested at each temperature (i.e. 4.2, 2.5 and 3.8 at 4 °C, 25 °C and 37 °C respectively).

in **Figure 3.6**. For this reason is safe to assume that as the suspending solution is highly acidic (i.e. $pH \in [2.5, 4.2]$) the CDs will be agglomerating, also due to the strong hydrogen bonds that can be formed among the surface groups.

As the solution starts to get slightly acidic, up to when it's slightly alkaline (i.e. $pH \in [5, 9]$), an incipient stability of CDs is observed, with zeta values ranging from $-10mV$ to $-30mV$: in this pH range most of the manipulations takes place. Eventually, in highly basic conditions (i.e. $pH \in [10, 12]$), the CDs shows a very good stability, ranging from $-32mV$ to $-50mV$, to even more negative values past a pH value equal to 12.

Now, to further investigate the ζ potential dependency on temperature, the other 2D plot deriving from the projection of the 3D data point this time on the $\zeta - T$ plane will be highlighted. This way, it will be obtained the plot on **Figure 3.5A**. It can be detected a linear trend in the temperature range investigated and the interesting outcome is that the slope of

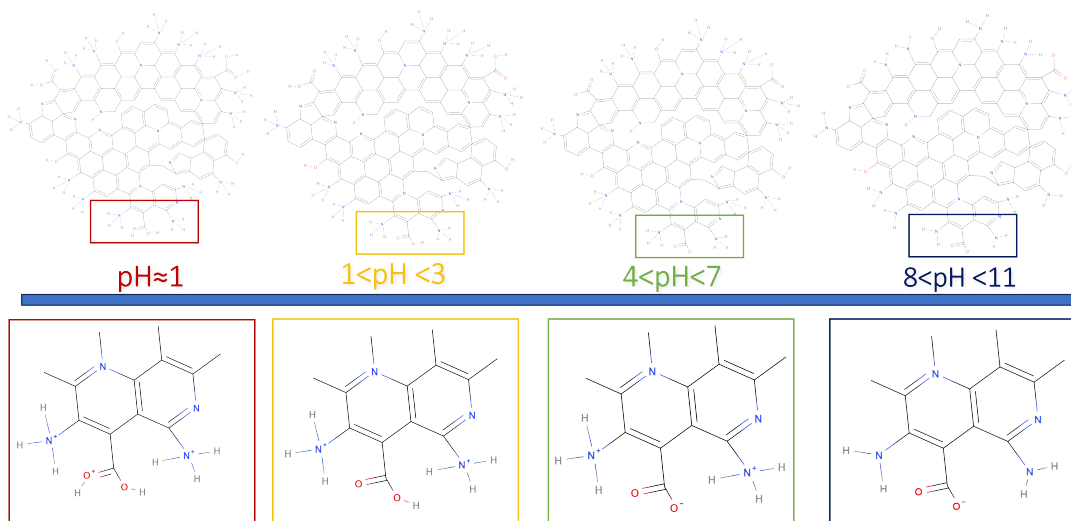


Figure 3.6: Possible scheme of surface charge variation at different pHs due to different degree of protonation/deprotonation of the surface groups exposed on the outer shells of the CDs.

the line best fitting the dataset also seems to show a trend.

As a matter of fact, $d\zeta/dT$ values below $\text{pH}=6$, where the *rapid agglomeration* band is placed do not seem to follow any pattern, while in the *incipient stability* range (i.e. between $\text{pH}=6$ and 9) the values seem to be around the zero line, or at least touching it with the error bar. Eventually, above $\text{pH}=9$, when the CDs seem to be found in a *good stability* band, the effect of temperature on zeta potential seems to be pretty clear: the derivative is strictly positive, and thus the zeta potential slightly increases as the temperature increases, meaning that a better dispersion is found at higher temperatures when the CDs are fully deprotonated.

Besides, the overall behavior can be explained by the fact that the stability is not the only occurring phenomenon in solution, as the external group will still be capable of forming pretty strong hydrogen bonds (due to N-H and O-H bonds being present on the shell), thus favouring a CDs assembling. This means that a proper ζ potential evaluation -especially in terms of temperature dependence- is hard to get when working in a *rapid agglomeration* regime. As soon as the CDs start to be more stable, their assembly is slowed down to a level which the temperature seems not to have an effect on their potential. Eventually, when they're totally stable in solution -still able to exert hydrogen bonds interactions- a proper evaluation of ζ potential dependency on temperature can be drawn.

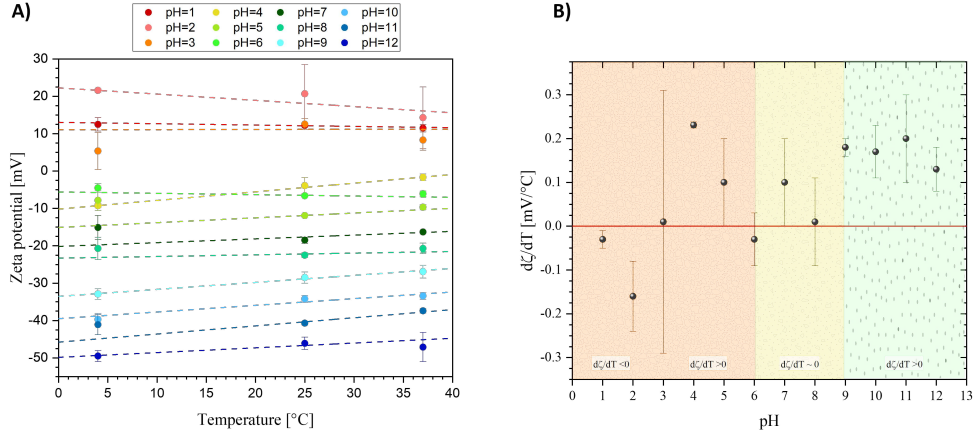


Figure 3.7: ζ vs T 2D plot at different pH values from the 3D plot of Figure 3.4. Dashed lines represent linear fits for all different pH values (A). ζ potential variation with temperature. Data are acquired from the linear fit and reported as a function of solution pH. The coloured bands represent the different colloidal stability zones taken from Figure 3.5, this time reported in the pH range typical for those zone. In **red** the *rapid agglomeration* zone, in **yellow** the *incipient stability* zone and in **green** the *good stability* zone (B).

3.3.3 Fourier-Transformed Infrared (FT-IR)

FT-IR analysis allowed us to infer some structural peculiarity of CDs, when compared to precursors (**Figure 3.8**). FA showed a quite complex spectrum, especially when compared to urea, which showed two more resolved peaks between 3450 and 3300 cm^{-1} corresponding to symmetric and asymmetric stretching of N-H bond, due to amine groups. These peaks seem preserved in the LT/CDs, but apparently not in MT and HT/CDs, where the wide zone going from 3400 to 2600 cm^{-1} is characteristic of a high-water retention in those samples.

Besides, when referring to C=O stretching it is stronger in FA and shows a stronger peak at 1686 cm^{-1} and a weaker at 1667 cm^{-1} in the fingerprint region, due to the high presence of these kind of C=O bonds along the structure in carboxyl groups and aromatic structures. In particular, when dealing with CDs, the only peak is the one at 1676 cm^{-1} , due to the presence of carboxyl groups on the outer surface of CDs.

Other representative peaks are those at 1602 and 680 cm^{-1} , representative of bending and wagging of amine group NH_2 , which are shifted to 1589 cm^{-1} in CDs samples: it can be noticed how for LT, MT and HT samples those peaks progressively decrease in intensity, due to the fact that the amine group decorating the outer shell of CDs decrease in number, as CDs get bigger in size at higher synthesis temperatures [30, 31].

Eventually, the presence of different peaks for C-N stretching in $1510\text{--}1410\text{ cm}^{-1}$ and $1310\text{--}1210\text{ cm}^{-1}$ ranges is representative of different heterocyclic structures in CDs, possibly due to the presence of C-N bonds in amine groups, as well as graphitic and pyridinic N [32]. In the end, the slight variation in intensity and position of C=C bond around 1400 cm^{-1} might represent the formation of graphitic structures in CDs due to carbonization reactions.

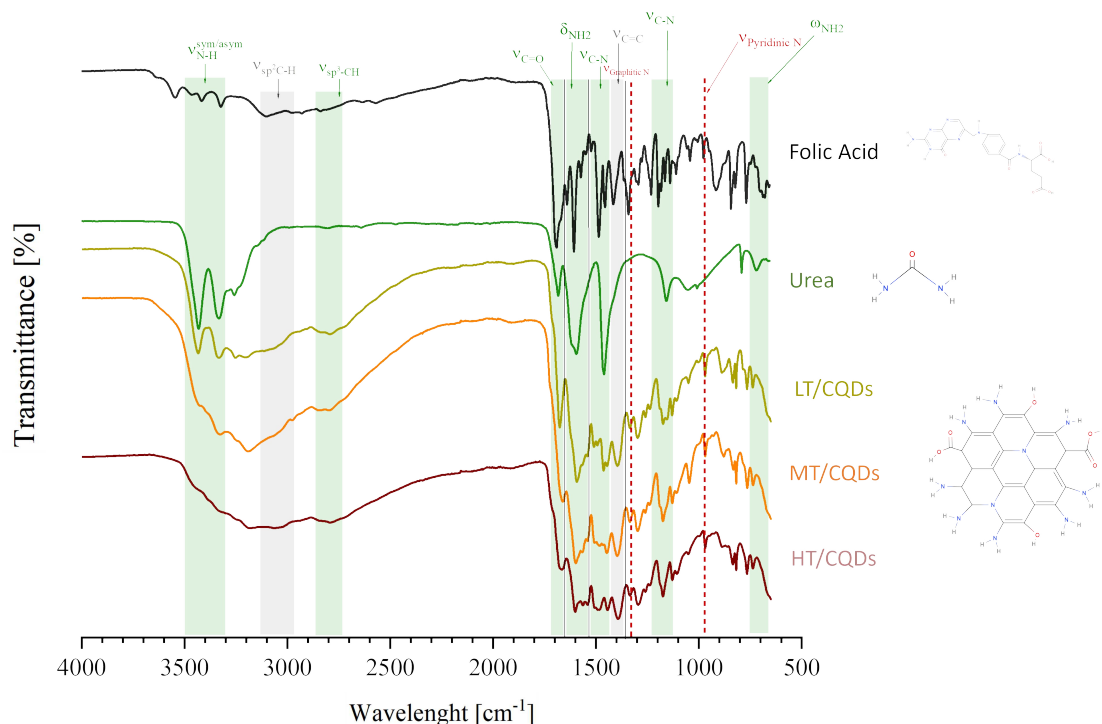


Figure 3.8: Fourier-Transformed Infrared (FT-IR) analysis of FA and urea precursors, as well as of FA-CDs synthesised in neutral conditions at different reaction temperatures (namely LT/CDs, MT/CDs and HT/CDs). In green peaks deriving from precursors are highlighted, while in grey are reported those strongly characteristic of CDs.

3.3.4 Thermogravimetric and Differential Thermogravimetric analysis (TGA and DTG) of precursors and CDs

TGA and DTG analysis allowed to interpret the thermal degradative pathways for FA and urea precursors, fundamental for synthesis understanding. In particular, urea showed a well resolved degradation path, where a first degradative region (from room temperature to $\sim 190^\circ\text{C}$) is associated with urea decomposition to different products, such as biuret, which, between $190\text{--}250^\circ\text{C}$, begins to decompose. Then, the third region, between 250 and $\sim 360^\circ\text{C}$,

is characterized by continued sublimation and decomposition of the remaining products, which are with good approximation totally eliminated at ~ 400 °C, reaching the total loss of mass [33] (**Figure 3.9A**).

On the other hand, FA does not completely degrade in the temperature window investigated. Besides, the decomposition can be summarized as occurring in three stages (**Figure 3.9B**). Firstly, around 100 °C the loss of adsorbed water takes place leaving behind the anhydrous sample, since this molecule can establish H bonds in several sites, binding water molecules. Then, the glutamic acid moiety is lost and thereby degraded around 234 °C. Then, FA becomes an amorphous mass around 374 °C [34]. A better visualization of precursor's degradation path can be found in **Appendix B**.

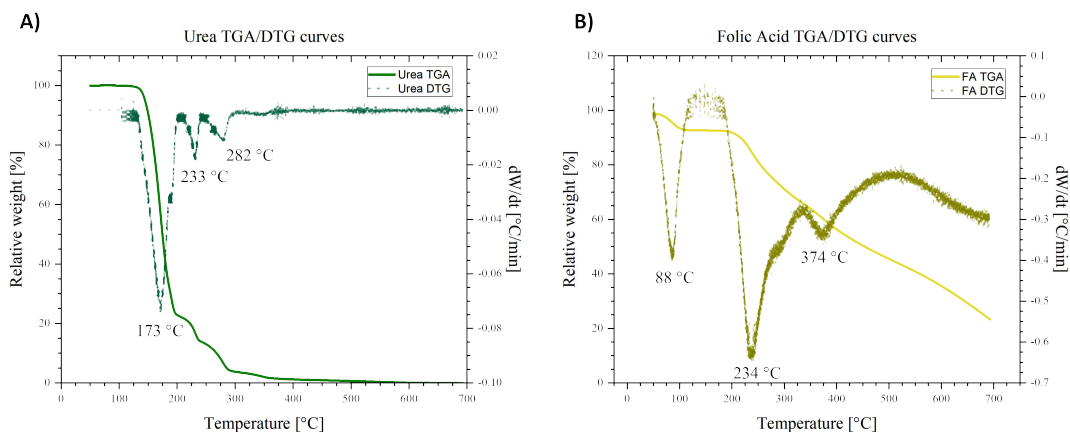


Figure 3.9: TGA and DTG curves of Urea (**A**) and Folic Acid (**B**) in the temperature range from 25 to 700 °C. DTG peaks are highlighted with the characteristic peak temperatures of degradation.

Once synthesis has occurred, the degradation spectra show some important differences, reflecting structural change in CDs depending on the synthesis temperature (**Figure 3.10**). The first difference that can be noticed is the fact that in a wider temperature window, the CDs behavior differ from one another. However, in all the samples the mass drop that ends at 1000 °C, signals complete decomposition of CDs to char.

More in general it can be seen how pretty much all the samples present some DTG peaks that resemble those from FA in terms of positions, but they are shifted towards lower temperatures. This suggests partial pre-degradation during microwave synthesis and can be attributed to the high-energy environment causing the breakdown of weaker bonds, forming

intermediates and new functional groups. These structural modifications may reduce thermal stability, leading to earlier degradation.

In particular, the LT/CDs showed a behavior that was more resembling the one of FA in **Figure 3.9B**, with the only difference that now the temperature range is widened and the material faces full degradation around 990°C.

The overall similarity of the LT/CDs TGA with the one from FA, together with other similarities (e.g. UV-Vis spectra in **Figure 3.11**) makes the hypothesis of a non-complete FA degradation plausible, or at least it allows to say that during the CDs formation, reagents didn't undergo severe structure-changing reactions.

On the other hand, HT/CDs show a quite similar trend. However, it cannot be said the same as for LT/CDs for this set of samples. As a matter of fact, HT/CDs' zeta size measurements (**Figure 3.3**), but also their oxidant and biological properties (**Figure 3.15**, **Figure 3.12**) show a strong difference compared to LT/CDs.

Therefore, the explanation for a similar behavior in thermal stability of these samples can be explained by means of a higher degree of carbonization and aromaticity. As a matter of fact, the samples treated at higher temperature show a smaller mass loss in the decomposition range of 100–250°C, revealing the efficient removal of oxygen-containing functional groups. Besides, the higher threshold to full degradation (around 1000°C) is representative of a very stable carbonous structure in the material, suggesting a high level of carbonization [35].

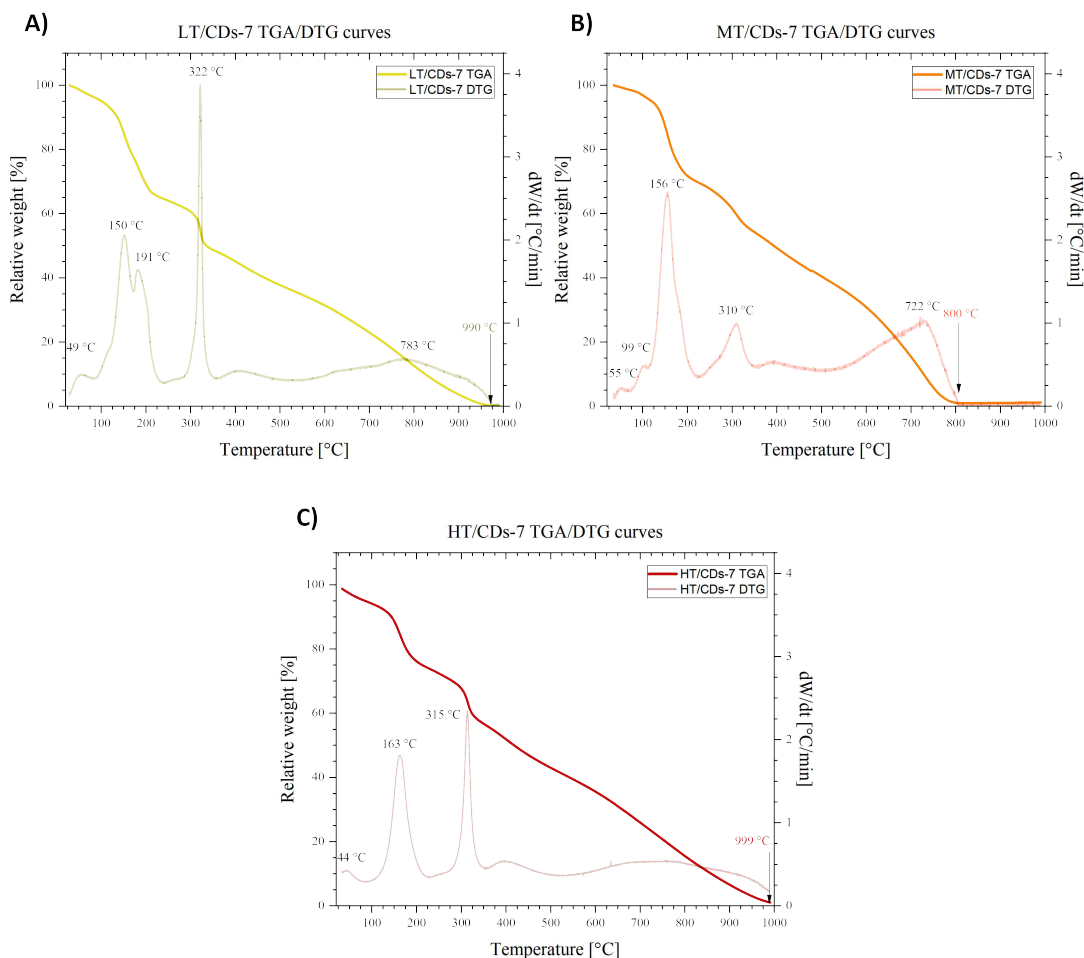


Figure 3.10: TGA and DTG curves of LT/CDs (A), MT/CDs (B) and HT/CDs (C) in the temperature range from 40 to 1000 °C. DTG peaks are highlighted with the characteristic peaks representing degradation temperatures.

3.3.5 UV-vis absorbances

UV-Vis spectra allow the identification of two main peaks (**Figure 3.11**). The absorption peaks between 250 and 300 nm, show the electronic transition $\pi \rightarrow \pi^*$, characteristic of the aromatic sp region, originated from the core of the CDs. The absorption peak from 325 to 400 nm, which marked the electronic transition $n \rightarrow \pi^*$ is attributed to the surface and edge defects of the carbon dots. Then, even though absorption peaks of LT, MT and HT/CDs had the absorption bands at same positions, however, the absorption intensities varied, indicating different carbon cores and surface chemical states [21].

In fact, the presence of urea in CDs, makes them rich in N, an n-type excitation atom on the surface will help increasing the n-type excitation fluorescence. However, too much N functionalization of FA-CDs might lead to an extreme passivation of their surfaces, with decreasing fluorescence properties [30].

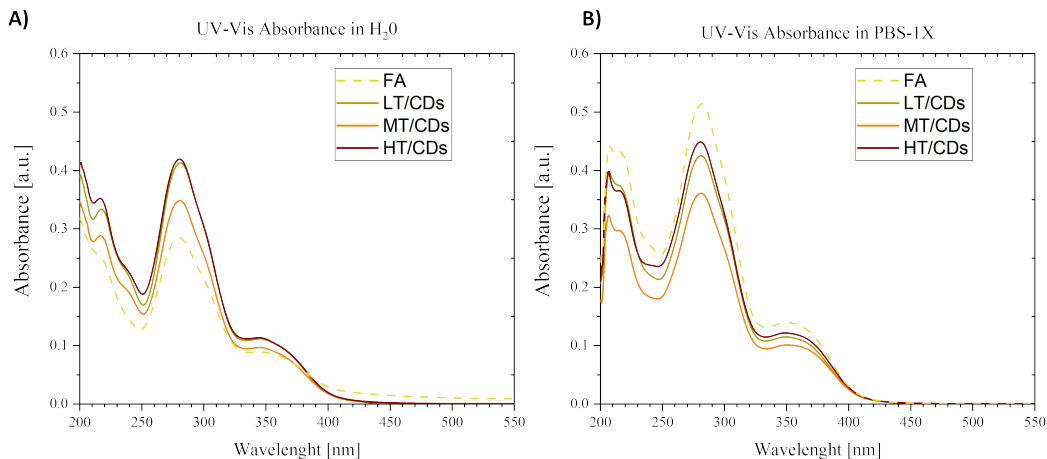


Figure 3.11: UV/Vis absorbance spectra of Folic acid, LT/CDs, MT/CDs and HT/CDs in distilled water (**A**) and in PBS 1X (**B**), at a concentration of 10 $\mu\text{g/ml}$.

3.3.6 NHLFs cells viability assay and morphological analysis

Cell viability and morphological analysis are a primary feature that must be evaluated in the design of CDs for biomedical applications. Testing a substantially large range of concentrations (from 50 to 1000 $\mu\text{g/ml}$), an extremely low cytotoxicity was shown by almost all the samples, apart from LT/CDs, which showed a slightly negative effect, especially at low concentration at first culturing day.

Moreover, for MT a different trend is observed with passing of time, where lower CDs concentrations behave as slightly toxic, but after 5 days this trend is inverted, as they become the most bio-compatible combination. The opposite phenomenon takes place with HT/CDs-7, where the slightly cytotoxic effect is confirmed after 4, 5 and 7 days. This result can be justified by providing two explanations strongly dependent one from the other: firstly, the higher amount of carbonization products, as shown by previous structural analysis, can notably hinder cell viability [36], and also the formation of oxidized functional groups, which may contribute to the generation of ROS, as shown in **Figure 3.15**.

Besides, the HT/CDs were reported to produce a significantly higher amount of ROS, even at low concentrations, which can surely negatively affect the long-term viability of cells. Overall, the AB viability assay shows that even at large doses, FA-based CDs obtained at different temperatures have little effect on the overall cell function making them potentially bio-compatible (**Figure 3.12**).

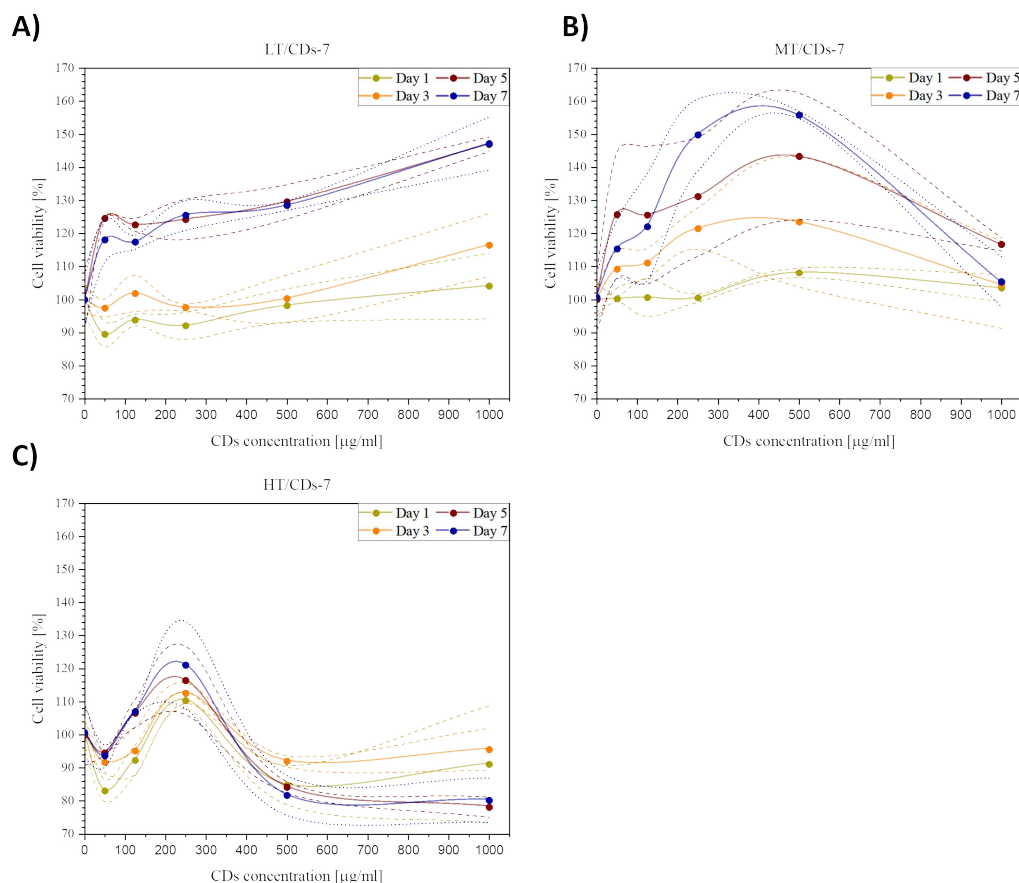


Figure 3.12: NHLF cells viability seeded with a different LT, MT and HT/CDs concentrations after 1 day (A), 3 days (B), 5 days (C) and 7 days (D) of culture. Dashed lines represent error bars B-spline connection.

Biocompatibility results were further confirmed by NHLFs morphology. Actin filaments, a constituent of the cytoskeleton, were stained with TRIC phalloidin after 7 days of incubation with systems. HLF cells, indeed, exhibited a typical non cytotoxic fibroblast-like morphology after the incubation with the formulations at different concentrations (**Figure 3.13**).

Their morphology was alike to the characteristic *in vitro* HLF morphology that is spread-shaped, as seen in control samples (at 0 μg/ml concentration), often characterized by several

extending processes, which consists of cell protrusions adhering at the flat surface. Overall, these results indicated that the devices have extremely good biocompatibility also after 7 days seeding, especially for lower concentrations of MT/CDs and medium to high concentration of LT and HT/CDs samples

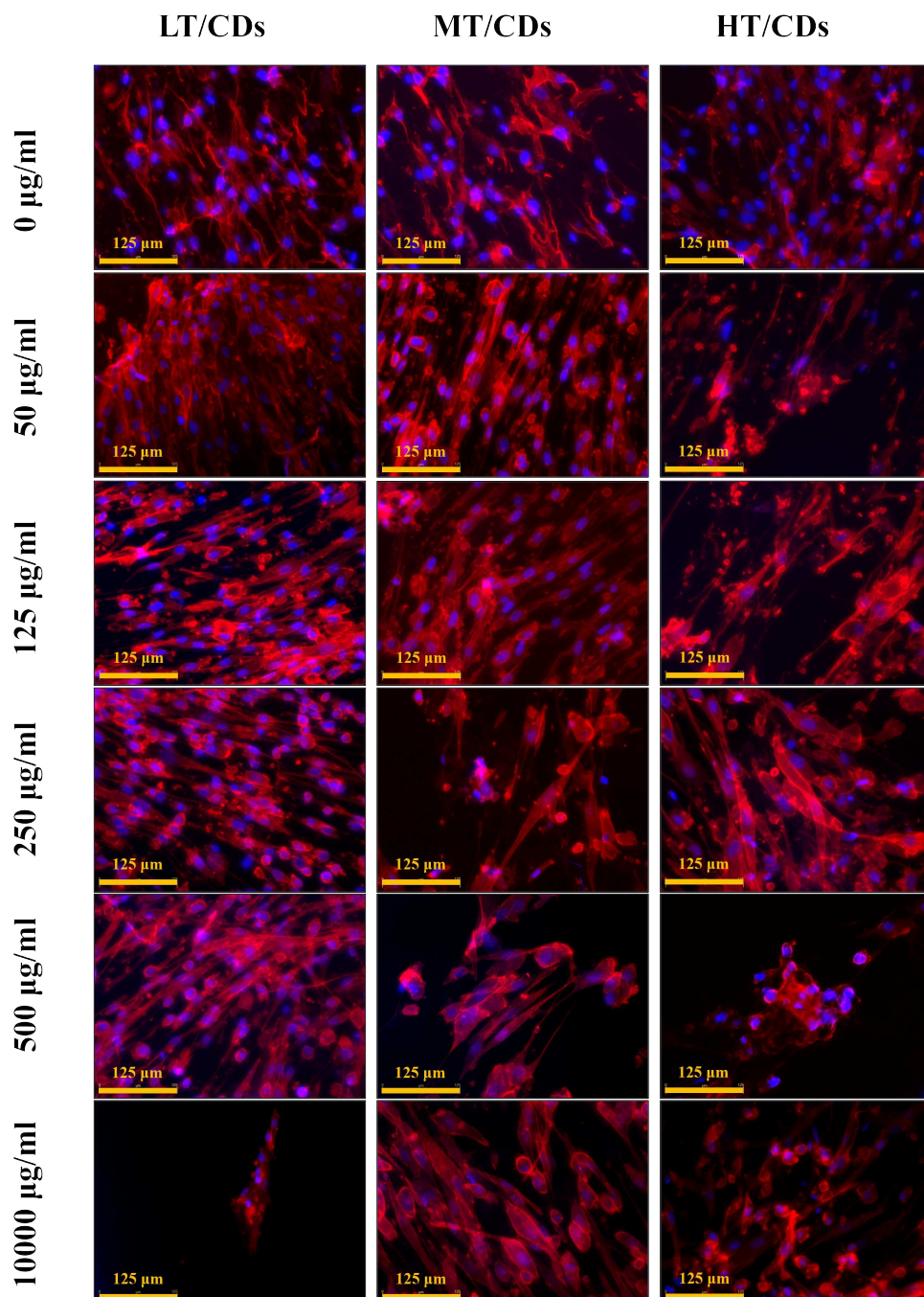


Figure 3.13: Morphological evaluation of HLF cells, assessed after 7 days of exposure with implemented media with different LT, MT and HT/CDs-7 concentrations. Nuclei were stained with blue DAPI and actin filaments in cytoskeleton were stained with red phalloidin. Scale bar: 50 μm .

3.3.7 Antimicrobial activity and oxidase-like activity by monitoring ROS generation

The antimicrobial tests have been performed on gram-positive SA and gram-negative PA, both strains involved in lung diseases, incubated with various concentrations (7500, 3750, 1875, 938, 469 and 234 $\mu\text{g/ml}$) of CDs obtained with the different reaction temperature. The results have been expressed as the percentage of bacterial growth compared to the non-treated bacteria (**Figure 3.14**).

The **Table 3.2** shows, for each synthesis protocol, the minimum concentration of product beyond which a decrease in bacterial proliferation occurs. Depending on the concentration and reaction temperature of CDs, different effects can be noted. Antimicrobial activity resulted efficient against the proliferation of gram-positive SA, while CDs did not significantly affect the proliferation of gram-negative PA.

In the case of SA, already at the lowest tested concentration (234 $\mu\text{g/ml}$), a reduction of 10% in bacterial growth occurred, except for LT/CDs which began to reduce bacterial growth starting from 496 $\mu\text{g/ml}$. Moreover, at higher concentrations, the most marked results were obtained for MT/CDs, for which a reduction of 40% in bacterial growth occurred at 938 $\mu\text{g/ml}$. At the highest concentration (7500 $\mu\text{g/ml}$), MT/CDs and HT/CDs reduced bacterial growth of 80%.

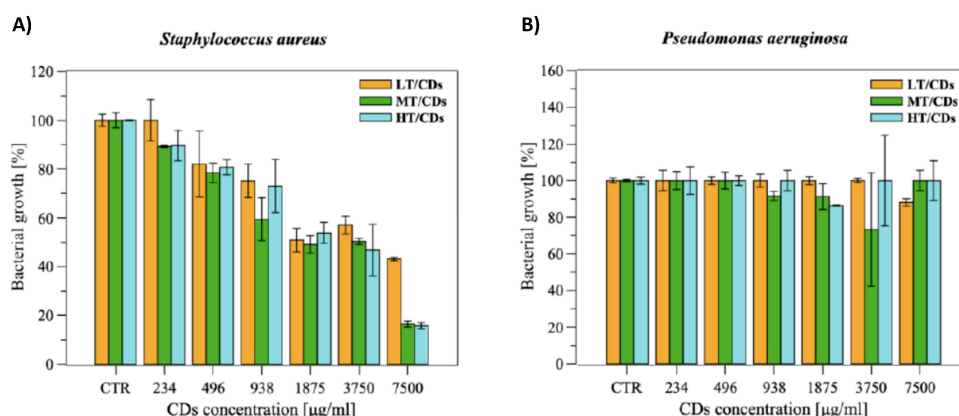


Figure 3.14: Percentage of bacterial growth of (A) gram-positive SA and (B) gram-negative PA after incubation of different concentration CDs. Percentage of bacterial growth was calculated compared to the non-treated bacteria and results are the mean of three independent experiments.

These results have proven to be truthfully promising, since CDs are capable of killing gram-positive bacteria, such as SA, having intrinsic antimicrobial capabilities, with the best performances attributed to MT/CDs. The antimicrobial properties of CDs could be ascribed to two different mechanisms: the first one due to generation of ROS which destroys bacterial membrane, killing them, and the second one due to CDs internalization thanks to their reduced dimensions, killing bacteria from the inside. ROS generation evaluation (**Figure 3.15**) showed that HT/CDs generates the highest quantity of ROS compared to LT/CDs and MT/CDs.

At the same time, probably, as the temperature increases, CDs size increases, as well as the degree of functionalization decreases, as high temperatures facilitate growth kinetics. For this reason, LT/CDs could be the smallest product and, therefore, the most easily internalized. Thus, MT/CDs could represent the right compromise between the two mechanisms, resulting the most effective in terms of antimicrobial activity.

Table 3.2: Minimum concentration affecting bacterial proliferation.

	Bacterial growth concentration [$\mu\text{g/ml}$]	
	<i>Staphylococcus</i>	<i>Pseudomonas</i>
	<i>aureus</i>	<i>aeruginosa</i>
LT/CDs	469	7500
MT/CDs	234	938
HT/CDs	234	1875

To explain the antimicrobial properties observed, results from UV-vis absorbance spectra of AA solutions when put in contact with FA or FA-CDs showed a concentration-dependent tendency to ROS generation, resulting in a decrease of AA characteristic peak at 266 nm (**Figure 3.15**). In particular, at a 17.5 $\mu\text{g/ml}$ concentration a slightly significant oxidation of AA (about 5%, corresponding to a normalised peak intensity equal to 94.8%) occurs when using FA or LT/CDs (p-value<0.05 vs CTR+).

On the other hand, a highly significant change in oxidation degree is observed when MT/CDs and HT/CDs are used, with a relative absorbance peak of ~ 85 and 84% (corresponding to an oxidation degree of ~ 15 and 16% respectively $p\text{-value} < 0.0001$ vs CTR+).

When the concentration is increased to $50 \mu\text{g/ml}$, further differences are highlighted among the different CDs samples. As a matter of fact, with the increasing concentration, FA still shows the lowest effect on AA oxidation ($15 \pm 2\%$) yet having a highly significant effect with respect to the positive control ($p\text{-value} < 0.0001$ vs CTR+). A slightly higher oxidation degree of $17 \pm 3\%$, although not significant with respect to FA, is shown with LT/CD-s (with a relative absorbance peak of $\sim 83\%$).

The greatest differences emerge when dealing with MT/CDs and HT/CDs. As a matter of fact, MT/CDs shows an oxidation degree of $28 \pm 2\%$, corresponding to a peak reduction around 72% , which result in a highly significant ($p\text{-value} < 0.0001$) effect with respect to CTR+ and very significant one to LT/CDs ($p\text{-value} < 0.01$). As for HT/CDs, the oxidation degree increases to a consistently significant value of $59 \pm 4\%$ (corresponding to a peak reduction around 41% , $p\text{-value} < 0.0001$ vs CTR+, FA, LT/CDs and MT/CDs).

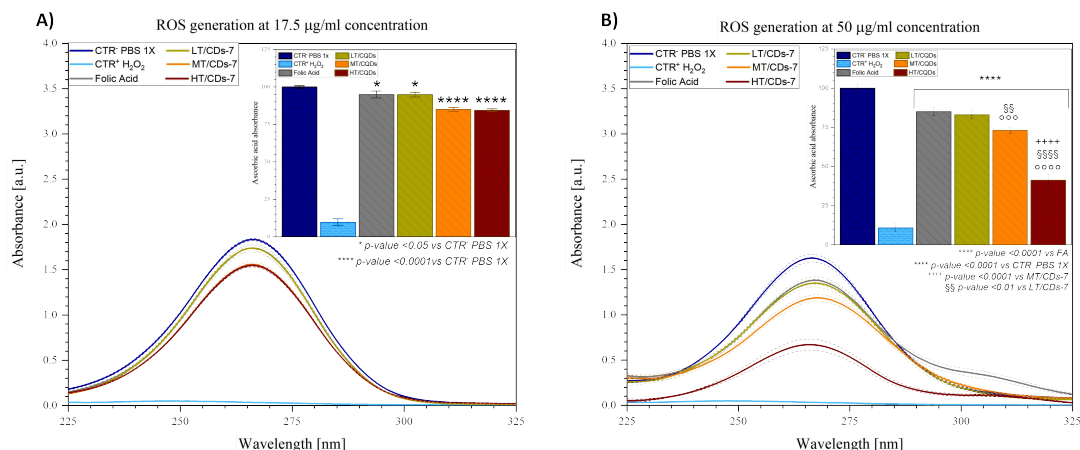


Figure 3.15: ROS formation in FA-CDs at two different CDs concentration: $17.5 \mu\text{g/ml}$ (A) and $50 \mu\text{g/ml}$ (B). The typical absorbance peak of L-Ascorbic acid is shown in PBS 1X and when put in contact with the FA-CDs solutions. PBS 1X represents the positive CTR, while H_2O_2 represents the negative CTR. In top right inserts the peak's intensity reduction is reported and linked to the oxidation degree of the molecule.

3.4 Conclusions and future perspectives

FA based CDs were synthesized by means of a simple one-step microwave-assisted method. In particular, three different reaction temperatures (130 °C, 170 °C and 185 °C) were investigated to study their influence on physicochemical properties and how such structural properties affect CDs functional properties in terms of biological response.

FT-IR spectra confirmed the formation of new chemical bonds respect to the precursors. Indeed, the shift of the peaks compared to the precursors, but also the relative intensity of the peaks between the CDs obtained with different protocols suggested a complex structure in the products presenting carboxyl and amino groups on their surfaces. Analogous observations can be made considering the UV-vis absorbance experiments.

Furthermore, UV-vis spectra of the different CDs confirmed the role of urea as additional source of nitrogen for surface functionalization. AB assay and cell morphology micrographs highlighted a good biocompatibility of all the products. In particular, the best choice in terms of biocompatibility was MT/CDs, especially for medium concentrations (300-500 $\mu\text{g/ml}$).

Finally, the antimicrobial activity evaluation also confirmed the greater efficacy of MT/CDs, which appeared to inhibit the proliferation of gram-positive bacteria (such as SA) even at low concentrations (234 $\mu\text{g/ml}$) thanks to a good compromise between size and ROS generation.

In the end, data from zeta potential and TGA performed on CDs, together with FTIR, UV-Vis and also biological results, allow to infer that the LT/CDs have a structure more resembling the one from FA itself, with a lower carbonization degree that enables a better cells viability at higher concentrations, but also a smaller degree of complex CDs formation and functional groups substitutions, showing a smaller antimicrobial activity.

On the other end, HT/CDs show a higher degree of carbonization -with higher thermal stability and lower isoelectric point- but also for this reason a consequent inferior bio compatibility compared to other samples, possibly in part due to the higher ROS generation. Then, MT/CDs seem to show the best compromise between functional groups distributions and degree of carbonization, able to provide a high NHLEs viability and a potent antimicrobial effect.

Contents

3.1 Introduction	72
3.2 Materials and methods	75
3.2.1 Microwave-assisted synthesis, purification and yield	75
3.2.2 Zeta potential measurements	76
3.2.3 Fourier-Transformed Infrared (FT-IR)	77
3.2.4 Thermogravimetric analysis (TGA) of precursors and FA-CDs	77
3.2.5 UV-Vis absorbance	77
3.2.6 Biological properties	78
3.2.7 Antimicrobial activity	79
3.3 Results and discussion	80
3.3.1 Microwave assisted CDs synthesis from FA and urea	80
3.3.2 Zeta potential measurement	81
3.3.3 Fourier-Transformed Infrared (FT-IR)	87
3.3.4 Thermogravimetric and Differential Thermogravimetric analysis (TGA and DTG) of precursors and CDs	88
3.3.5 UV-vis absorbances	91
3.3.6 NHLFs cells viability assay and morphological analysis	92
3.3.7 Antimicrobial activity and oxidase-like activity by monitoring ROS generation	95
3.4 Conclusions and future perspectives	98

Chapter 4

Design and optimization of a 3D biomimetic alveolar lung tissue construct

Contents

4.1 Introduction	102
4.2 Materials and methods	106
4.2.1 Materials	106
4.2.2 Cells culture	106
4.2.3 Alginate microbeads (mAlgs) fabrication and characterization	107
4.2.4 Collagen/Hyaluronic Acid hydrogel for 3D alveolar construct	110
4.2.5 mAlgs seeding density optimization in Alexa-Fluor-488 labelled Col/HA hydrogel and 3D alveolar construct characterization	110
4.2.6 Fibroblasts seeding density optimization in 3D alveolar constructs	111
4.2.7 Rheological characterizations	111
4.2.8 Coculture characterization in 3D alveolar construct	114
4.3 Results and discussion	117
4.3.1 mAlgs synthesis and characterization	117
4.3.2 mAlgs seeding density optimization	121

4.3.3	Effects of EDTA treatment: mechanical properties and NHLFs viability	123
4.3.4	NHLFs seeding density optimization and viability in time	124
4.3.5	A549 encapsulation in mAlgs	126
4.3.6	3D coculture system optimization	129
4.3.7	Inflammatory insult by PMA treatment: effect on alveolar walls for- mation	133
4.3.8	Inflammatory insult by PMA treatment: effect on cytokines expres- sion, NHLFs activation and fibrotic response	137
4.3.9	Conclusions and future perspectives	144

4.1 Introduction

Respiratory diseases -especially chronic diseases like COPD, asthma, pulmonary fibrosis, etc.- in 2017 accounted for around 544.9 million estimated cases of illnesses worldwide, representing a 39.8% rise compared with the number of individuals affected in 1990 [1].

Now, as an example neither asthma nor COPD can be cured, but palliative treatments can reduce symptoms, preventing deterioration and slightly improve daily life [2]. In a similar way also bacterial and viral infections, or ALIs, including ARDS, pose significant clinical challenges.

As a matter of fact, the complexity of inflammatory responses and tissue remodeling processes in these conditions makes it difficult to predict and optimize therapeutic responses. The main reason for that resides in the specific limitations in the testing platform, as the need for advanced *in vitro* models to investigate lung diseases and assess new therapies has become increasingly evident.

Traditional 2D cell culture models fail to capture the structural and physiological characteristics of the *in vivo* lung microenvironment, limiting insight into the cellular and tissue processes involved, like cell-to-matrix interaction or specific crosstalk between cells populations [3]. Specifically, the lack of a three-dimensional architecture, different cell populations and their realistic co-localization of diverse reduces the accuracy of results, often complicating the translation of laboratory findings to clinical studies.

From this point of view an alternative is proposed by *in vivo* models, where the complex physiological response is reached, with a proper communication also with other districts, like liver, hearth, brain, ect. However, these models not only face ethical concerns and long experimental times, but sometimes they also show limitations in replicating human-specific disease mechanisms [4]. This is due to the difference in animals physiology and the use for example of immunodeficient mouse models in which the immune system has been impaired to increase susceptibility to infections, or to similar diseases [5].

On the other hand, lung-on-chip platforms can provide dynamic flow conditions and better replicate the biomechanical forces experienced in the lung [6]. However they are often

limited in cellular and architectural complexity and scalability, which can reduce their predictive capacity for therapeutic testing.

For this reason, organoids offer a promising approach by mimicking cell differentiation and structural features of lung tissue. They can be defined as three-dimensional cell cultures obtained from MSCs or iPSCs that does not require the attachment of cells to a flat substrate but can be seen as complex cell clusters from different cell origin that self-organizes to exhibit a 3D structure resembling with good approximation the native organ. The cells are embedded in a gel made of a complex mixture of different ECM proteins.

In the field of lung tissue regeneration, among the main advantages of organoids, their reliable nature as models to investigate pulmonary injury and repair mechanisms stands out, since allows cellular inter-action. To date, several works have faced the production of lung tumour organoids, but few of them investigated healthy organoids, deepening a study of nanoparticles internalization and interaction with these constructs. Their use is actually regarded as a new approach in toxicity testing of nanotherapeutics [7, 8, 9].

However, one of the mainly exposed limitation of organoids, it is the unreached requirement of a stable culture within an air-liquid interface, to mimic an alveolo-capillary barrier. More in general, they often lack the spatial organization and precise cellular architecture of the alveolar environment and from the experimental point of view, their response sometimes is barely reproducible, as they usually also rely on the presence of matrigel as ECM-like component, which have also been documented to have high batch-to-batch variability [10].

In this context some recent advancements in 3D models have significantly impacted regenerative medicine and lung tissue engineering, aiming to more accurately mimic the lung's native environment and improve therapeutic outcomes [11, 12]. Recent cutting-edge 3D culture systems use biomimetic scaffolds composed of ECM-like natural polymers such as Col, HA, etc. to support cellular organization and alveolar structure. By recreating the spatial arrangement and matrix components of lung tissue, these scaffolds facilitate cell differentiation and promote behaviors that closely resemble those of human lung cells, enhancing the model's physiological relevance as advanced tools for drug testing, disease modeling, and the development of targeted lung therapies.

In addition to that, novel approaches have been developed in 3D bioprinting or microfluidic technologies, allowing a better standardized the fabrication of highly structured and tunable platforms endowed with an good reproducibility [13].

However, few limitations are shown in current models, as they often fall short in recreating the complex interplay between different cell types, particularly the coculture of fibroblasts and epithelial cells, which is essential for accurately mimicking the lung microenvironment [14]. Many current models fail to provide a comprehensive understanding of the dynamic relationships between the ECM components, 3D architecture, and the spatial colocalization of various cell types [15]. This disconnect hinders the translation of experimental findings to clinical settings, as it does not adequately reflect the physiological conditions present *in vivo*.

For this reason, the proposed biomimetic model integrates all these critical elements, allowing for a more holistic representation of lung tissue. By incorporating both human lung fibroblasts (NHLFs) and epithelial cells (A549) within a carefully designed 3D matrix, this model can replicate the structural and functional characteristics of healthy lung tissue.

Briefly, the experimental procedure involved the emulsification of an alginate solution droplets containing A549 cells, which were then integrated into a NHLFs-loaded Col-HA matrix, ensuring cell viability during extended culture periods, as shown by ATP and live/dead assays. This biomimetic encapsulation within the ECM-like matrix created a stable 3D environment that, after hydrogel dissolution, facilitated a complex network for cellular interactions and spatial organization of both cell types. Unlike previous models, which often lacked clear colocalization of fibroblasts and epithelial cells, this system successfully established a multicellular alveolar construct with defined cellular positioning, as proven by confocal acquisitions after cell labelling with PKH26 and CFSE staining.

Additionally, the 3D alveolar model demonstrated its versatility as a platform for studying the responses of distal lung tissue under both healthy and inflamed conditions, particularly through treatment with phorbol myristate acetate (PMA) to mimic inflammation. The model's responsiveness to inflammatory stimuli was monitored by ELISA kits for pro-inflammatory cytokines expression and immunostaining for specific activation markers. This showed its utility for investigating pathophysiological processes such as ECM remodeling and cell activation

within a more realistic, *in vivo*-like environment. This characteristic positions the system as a research tool for exploring lung pathologies and also as a test bed for experimental therapies targeting inflammatory pathways, cellular cross-talk, and tissue repair in alveolar settings.

The ability to observe cellular interactions and responses in such a realistic setting enhances the potential for studying disease mechanisms and therapeutic interventions. This innovative approach not only has the potentiality to improve the understanding of lung biology but also paves the way for more effective strategies in lung experimental treatments.

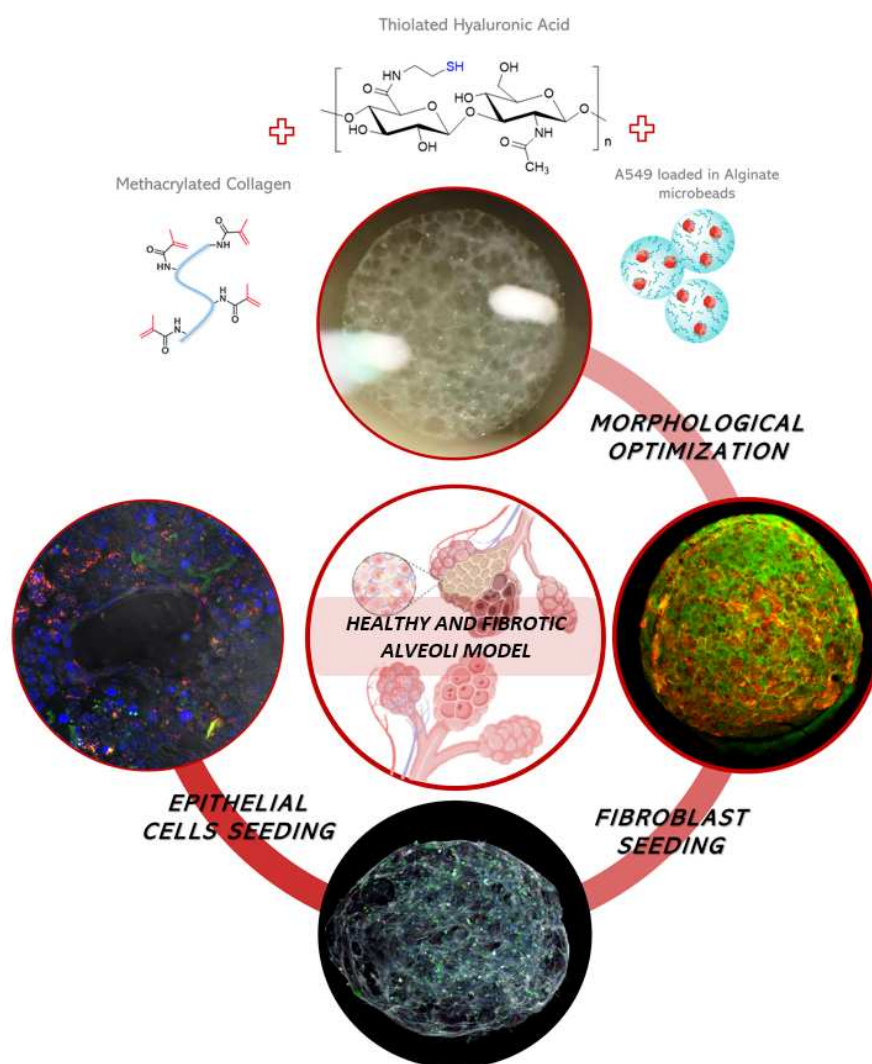


Figure 4.1: Representative scheme of this study. A 3D biomimetic alveolar model was developed, starting from a scaffold composed of ECM-like materials (i.e. methacrylated Col and thiolated HA). The scaffold was first optimized in terms of alveolar architecture, involving the dissolution of alginate microbeads (mAlgs). Then, it was first optimized the seeding of stromal cells (i.e. NHLFs) and after that also the seeding optimization of epithelial cells (i.e. A549) was addressed. Eventually, a fully working, 3D biomimetic coculture system with colocalized cell types was obtained and used as a promising model for healthy and fibrotic alveoli, following PMA insult.

4.2 Materials and methods

4.2.1 Materials

PhotoCol Methacrylated Collagen Type I (Cat #5198) and Heprasil Thiol-Modified Hyaluronin/Heparin (Cat #GS215) were purchased from Advanced Biomatrix. . Irgacure, 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone was purchased from Sigma (Cat #410896), together with Alginic acid sodium salt (Cat #180947), while EDTA solution 0.5 M was purchased from Fisher Scientific. FluoSpheres™ Carboxylate-Modified Microspheres were purchased from Thermo Fisher Scientific (Cat #F8819).

Primary normal human lung fibroblasts were purchased from Lonza (NHLFs, CC-2512), while adenocarcinomic human alveolar basal epithelial cells were purchased from ATCC (A549, CCL-185).

Human Simple-Step IL-6, IL-8, IL-1 β and TNF α ELISA Kits (ab178013, ab214030, ab214025, ab181421) were purchased from Abcam, together with, Picro Sirius Red Stain Kit (Cat #Ab150681), while Alcian Blue solution was purchased from Sigma (Cat #B8438). Alexa Fluor-647 Phalloidin was purchased from Thermo Fisher Scientific (A22287).

4.2.2 Cells culture

Normal Human Lung Fibroblast (NHLFs) primary cells were cultured in Fibroblast Growth Medium-2 BulletKit (FBM, Lonza) containing 2% FBS. Instead, lung carcinoma epithelial cells that constitute a cell line, A549, was grown in Dulbecco Modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 1% Penicillin-Streptomycin. All cell types were grown in a T-75 flask at 37°C and 5% CO₂.

Primary cells were used up to passage 9 and A549 cells were used until passage 11. All cells were passaged with 0.05% Trypsin/EDTA around 85% confluency and resuspended in their appropriate media for counting prior to use in hydrogel construct experiments. Cells were counted after trypan blue staining (NanoEntek) by means of a Bürker-Türk counting chamber (Sigma). Media was replenished every 2 days for NHLFs cultures, while every 3 days for A549.

Bronchial Epithelial Cell Growth Medium BulletKit (BEBM, Lonza) was used in a 1:1 ratio with FBM to culture the produced 3D tissue coculture constructs.

A549 and NHLFs fluorescent labeling by PKH26 and CFSE staining

For PKH26 staining of A549 cells, a pellet of 5×10^6 cells was resuspended in 250 μ l dilution solution. To this, 1 μ l of PKH26 staining solution in 250 μ l dilution solution was added. After incubating for 5 minutes at room temperature, 500 μ l of FBS was added to halt the reaction, followed by centrifugation at 300 rcf for 5 minutes and three washes with culture medium.

Similarly, NHLFs were stained with CFSE. A pellet of 5×10^6 cells was suspended in 500 μ l of PBS with 0.5 μ l of FBS. Then, 0.5 μ l of 10 μ m CFSE was added, and the cells were incubated for 10 minutes at room temperature. After adding 500 μ l of FBS to stop staining and incubating for 10 minutes, the cells were centrifuged at 300 rcf for 5 minutes and washed three times with medium.

4.2.3 Alginate microbeads (mAlgs) fabrication and characterization

Blank and fluorescently labeled mAlgs fabrication

In order to produce mAlgs, a bulk synthesis was adopted through the formation of an emulsion, followed by an external gelation technique [16]. Therefore, alginate was preliminarily dissolved in Milli-Q water to a final concentration of 2% w/v and 3% w/v as stock solutions. That way it was formed the water phase to produce the blank mAlgs, while Nile-red labelled FluoSpheres™ were used at a 1:100 dilution in Alg solution to fluorescently tag the mAlg for further experiments ¹.

¹This specific FluoSpheres™ dilution was obtained after an optimization procedure, selecting the dilution that would allow the precise identification of the mAlg shape once observed at the confocal microscope.

For each synthesis 2ml of water phase were used. Then, Span80 was used as surfactant for emulsion stabilization and dissolved to a final concentration of 0.5%v/v into 20 ml of rapeseed oil to form the oil phase². Eventually, 25 ml of a 360 mM $CaCl_2$ solution (corresponding to a 4%w/v solution) was prepared to act as a gelling solution³.

The oil phase was subjected to a constant stirring at 600 rpm, while the water phase was added dropwise through a 30G syringe. The emulsion was let for few minutes under constant stirring and then, it was slowly poured on top of the gelling $CaCl_2$ solution and slowly stirred at 200 rpm for 10 minutes, to allow complete gelation. Eventually a 70%v/V ethanol solution was added on top of the suspension and few more minutes were waited, to allow the emulsion breaking, helping the separation of the two phases.

The water phase -now containing mAlg in suspension- was collected and centrifuged at 200 rcf for 5 minutes to collect the mAlgs. Then 2 washes in PBS 1X ($-CaCl_2$ and $-MgCl_2$) were performed and the pellet was furtherly filtered using a sterile cell strainer (with a 40 μm cutoff). Briefly, the suspension was poured on top of the cell strainer, posed on top of a 50 ml Falcon tube. The system was covered with Parafilm and quickly spinned for 5s to allow the water to pass through the filters, together with mAlgs to small for the interest of this research⁴. At this point mAlgs were stored at 4°C for further use or characterization.

Epithelial cells encapsulation in mAlg

In order to produce an epithelial cell loaded mAlgs suspension, a sterile 2% alginate solution was obtained by alginate dissolution in PBS 1X ($-CaCl_2$, $-MgCl_2$) and a further autoclave sterilization at 121°C for 30 mins. Then, the A549 cells were trypsinized, pelleted and resuspended in the sterile alginate solution as the final emulsion water phase. As a matter of fact, the overall synthesis procedure remained the same (an emulsion method and gelification through external gelation) but some precautions had to be taken, in order to keep the whole procedure bio-compatible (see **Appendix D**).

²Span80 shows an HLB (Hydrophilic-Lipophilic Balance) value equal to 4.3 and therefore is suitable for water-in-oil (W/O) emulsions. Besides, its CMC in most of organic oils is around 0.5%v/v, so the chosen concentration allowed for sure the formation of micelles in the oil phase.

³In general it is suggested to prepare a $CaCl_2$ solution that is at least 10 times the volume of Alg solution, in order to maximize the yield [17].

⁴As a matter of fact, mAlgs smaller than 40 μm were not of interest, since the desired porosity had to be at least around 50 μm . Besides, the centrifugation allowed to leave a very little amount of water between the mAlgs to allow further re-suspension.

First of all, no ethanol can be used for emulsion breaking, due to its cytotoxic effect. Furthermore, a different gelling solution had to be used, consisting of a 1:1 PBS 1X-complete DMEM mixture with a final concentration $[CaCl_2] \sim 181mM$ ⁵. Such solution was left to gel mAlgs for a longer time (15 minutes) under a less intense stirring velocity (usually below 150 rpm). Then, the suspension was centrifuged for 5 mins at 200 rcf and washed twice with culture media. Eventually, system was covered with sterile Parafilm and quickly spun for 5s to allow the water to pass through the filters, as in the blank mAlg fabrication route. The A549-loaded mAlg were immediately used for further experiments or resuspended in DMEM in a T-25 Non-treated Flask and left under gentle constant shaking at 37°C.



Figure 4.2: Representative scheme of fabrication route of A549 loaded mAlgs by external gelation method in a bulk emulsion.

mAlgs size distribution characterization

Optical microscopy with a 4X objective was used to run a dimensional analysis of blank and A549-loaded mAlgs after synthesis. Then, acquired images were imported into ImageJ software (NIH, Bethesda, MD, USA) for post-processing analysis and quantification of mAlgs diameter.

Briefly, a statistic was made by analysing a minimum of 300 microbeads. For each sample, all the significant statistical parameters (first three quartiles and IQR, the interquartile range) were reported in terms of a boxplot using Matlab programming platform. and a statistical analysis was run with the one-way analysis of variance (ANOVA) and a $p - value < 0.0001$ was considered significant.

⁵The 1:1 mixture PBS and DMEM was used in order to dilute the proteins dissolved in the media that could further stabilize the emulsion, allowing for a faster phase separation

4.2.4 Collagen/Hyaluronic Acid hydrogel for 3D alveolar construct

Fibroblasts, mAlgs or r-mAlgs were suspended in hydrogel precursor solutions consisting of different concentration of methacrylated collagen I and thiolated hyaluronic acid. Briefly, for all formulations thiolated hyaluronic acid (Advanced Biomatrix, Carlsbad, CA) was reconstituted at 1 mg/ml in sterile, degassed water containing 0.01% w/v photoinitiator (2-Hydroxy-40-(2-hydroxyethoxy)-2-methylpropiophenone, Sigma, St. Louis, MO). Methacrylated collagen I (Advanced Biomatrix) was dissolved in sterile filtered 20 mM acetic acid to a final concentration of 6 mg/ml at 4 °C.

Immediately before mixing hydrogel precursors, collagen I aliquots were neutralized with a sodium hydroxide neutralization solution (Advanced Biomatrix). The collagen and hyaluronic acid solutions were then combined at a 3:1 ratio and used to further resuspend mAlgs, cells or a combination of them.

4.2.5 mAlgs seeding density optimization in Alexa-Fluor-488 labelled Col/HA hydrogel and 3D alveolar construct characterization

In order to preliminary study the optimal pores distribution in the 3D alveolar structure, a covalent staining of Col/HA hydrogel was obtained by a treatment with maleimide functionalized Alexa Fluor-488. Briefly, 0.8 μ L of Alexa stock solution were dissolved in 100 μ L of Col/HA hydrogel solution. Then, r-mAlgs were seeded in the fluorescent labelled hydrogel precursors at different ratios.

The composite hydrogel was then pipetted in 5 μ L droplets onto PDMS-coated 96-well treated round bottom plates⁶. Then, the green fluorescent composite hydrogel was left 10 min in incubator at 37°C to allow the maleimide-thiol reaction⁷ and then it was crosslinked with a 365 nm UV LED light (BlueWave RediCure 365, Dymax, Torrington, CT) for 2 s. The 5 μ L composite hydrogels were cultured in 300 μ L of conditioned media per well.

⁶The use of PDMS in this and other studies allowed the formation of an hydrophobic surface on which the droplets could maintain as well as possible a round shape, that kept three-dimensionality of the system also when scaled-down.

⁷This reaction is favoured and the reaction time wasn't prolonged in order to be sure to not saturate a high amount of thiol groups on the HA, ideally keeping a sufficient amount of those groups free to react with the methacrylated moieties of collagen. As a matter of fact, the maleimides are far more reactive than the methacrylates[18].

4.2.6 Fibroblasts seeding density optimization in 3D alveolar constructs

Then, from the mAlgs seeding optimization experiment, a 3:5 mAlg:Col/HA ratio was chosen, to further optimize NHLFs seeding in the 3D construct. Briefly, two different cell densities were investigated: 5×10^5 and 8×10^6 cells/mL, corresponding to 2×10^3 and 4×10^4 cells/construct respectively.

Then after selecting the second seeding density, a preliminary EDTA treatment was run on the 3D constructs to monitor the evolution in time (after 4, 7 and 14 days) by confocal microscopy. Briefly, r-mAlgs were seeded in the Col/HA precursors solution, together with CFSE-labelled NHLFs pellet and after a gentle mixing, the $5\mu\text{l}$ droplets were obtained on the PDMS-coated 48-wells plate. After 1 day of culture in culture media, the 1hr EDTA 0.01 M treatment was performed on the samples, while untreated samples were used as controls.

3D alveolar systems were cultured up to 14 days and subsequently fixed with 4% Paraformaldehyde (PFA) overnight at 4°C and further counterstained with Alexa Fluor-647 Phalloidin and DAPI. Then, fluorescent images were taken with a Nikon AXR Confocal microscope (Nikon, Melville, NY).

Once optimized both the NHLFs seeding density, as well as the mAlg:Col/HA seeding ratio, the design parameters for the constitution of the final 3D alveolar coculture system were set. In particular the 8×10^6 cells/ml density was used for NHLFs, and the 3:5 ratio (corresponding to 200 mg/ml) of A549-loaded mAlgs, seeded with a 20×10^6 cells/ml A549 cells density (**Figure 4.3**).

4.2.7 Rheological characterizations

Rheology was used not only as a tool to test mechanical properties of the made 3D alveolar constructs following specific treatments, but it has also been used as a guide to optimize the fabrication route across this work.

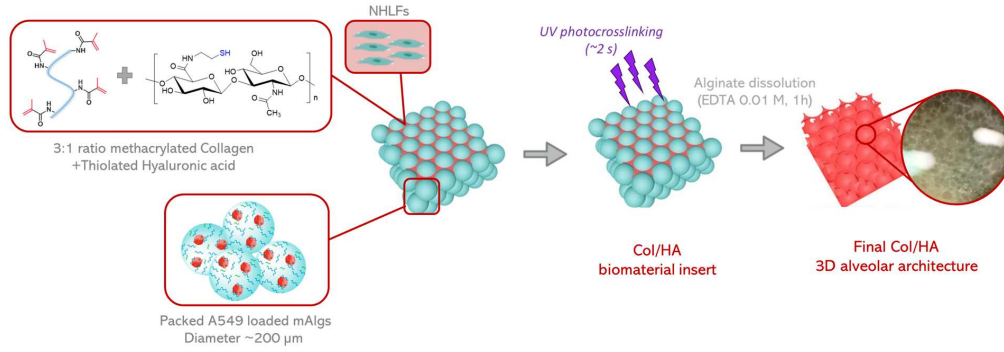


Figure 4.3: Representative scheme. Fabrication of 3D biomimetic alveolar constructs by different seeding procedure of epithelial cells (A549) and normal human lung fibroblasts (NHLFs) in a biomaterial insert, with further dissolution of mAlgs by 0.01M EDTA.

Alginate solution and oil phase characterization

Rheological analysis were conducted on alginate 2% and 3% solutions and compared with rapeseed oil (RO), in order to get preliminaries information on the viscosity ratio $p = \frac{\eta_{Alg}}{\eta_{RO}}$ between the water and the oil phase. In order to do so, a steady shear flow tests at 37°C was performed with a C60/1° CS L cone geometry by means of a MARS rheometerTM III, HAAKETM (TermoFisher Scientific, Waltham, MA, USA).

Alginate 3D gels

Rheological analysis (Discovery HR10 rheometer, TA Instruments) were conducted on 3D alginate samples at 2% and 3% w/v with varying $-CaCl_2$ concentrations, to test the mechanical properties of the single mAlg, scaled up to a 3D puck. Briefly the alg solutions were poured in a 24 well plate and were soaked overnight under constant agitation.

The gelling solution for the first set of experiment was a 360 mM $-CaCl_2$ solution was used, to mimic with good approximation the gelation of blank mAlgs. Then, to study and optimize the gelation mechanism in A549-loaded mAlgs, 3D gels were gelled in a PBS 1X-complete DMEM 1:1 mixture, in order to optimize the best $-CaCl_2$ concentration to obtain a gel⁸.

The tests these samples underwent strain sweeps from 0.01% to 50% at 1 Hz and fre-

⁸This experiment was paired with a live/dead assay to be sure that the A549 inside the mAlgs were still viable

quency sweeps from 0.01 Hz to 50 Hz, under a 0.7% strain at 37°C.

The objective was to get information about the gel's *mesh-size* (ξ), in order to have a more efficient EDTA treatment, minimizing cells' death. The mesh-size was then estimated using elastic blob theory, which is appropriate for swollen network⁹ as:

$$\xi = \left(\frac{k_B T}{|G_0^*|} \right)^{\frac{1}{3}} \quad (4.1)$$

where, $|G_0^*|$ is the plateau complex modulus (in Pa), $k_B = 1.38 \times 10^{-23} J/K$ is the Boltzmann constant and T is the absolute temperature [19].

mAlgs suspension characterization

A further analysis was conducted on the mAlg suspension resulting from the last step of the purification, once retrieved the sample from the cell strainer. Those analysis were conducted using a Discovery HR10 rheometer (TA Instruments) and aimed to investigating the thixotropic behavior of the mAlg suspension and will be discussed in **Appendix D**.

Two types of tests were performed: steady shear flow tests and oscillatory modulus recovery tests, all conducted at 25°C. For the steady shear flow tests, a 20 mm cone-plate geometry was used. Flow curves were measured with a shear rate range from 0.1 to 700 s^{-1} , followed by looped shear rate ramps from 1 to 150 s^{-1} . Each ramp lasted 300 s, allowing the characterization of the material's shear thinning and thixotropic behavior.

Oscillatory modulus recovery tests were conducted using a 20 mm parallel-plate geometry. The test involved three stages, where frequency was set constant to 1Hz:

- A linear viscoelastic regime ($\gamma = 1\%$) for 150 s to establish the baseline storage (G') and loss modulus (G'');
- A non-linear regime ($\gamma = 100\%$) for 90 s to induce structural breakdown;
- A recovery phase in the linear viscoelastic regime ($\gamma = 1\%$) for 300 s to assess the recovery of the moduli after the shear stress was removed.

⁹This model assumes that the polymers [3] behave as elastically active chains, and the mesh size of the gel corresponds to the dimensions of the elastic blob.

The tests allowed the evaluation of the material's ability to recover its structure after deformation, providing insights into its thixotropic and viscoelastic properties.

3D alveolar constructs characterization

To investigate the rheological properties of 3D cylindrical alveolar pucks (8 mm diameter, ~ 1 mm thick) from gelified thiolated HA and methacrylate Col in PDMS molds, a series of tests were conducted at 37°C.

In particular, the analysis focused on two formulations: Col/HA systems and those loaded with mAlgs at a 3:5 ratio, before and after EDTA treatment. Strain sweeps were performed from 0.01% to 50% at 0.3 Hz, followed by frequency sweeps from 0.01 Hz to 50 Hz at 5% strain.

Additionally, rheological analysis were conducted at 37°C on 3D alveolar samples before and after the EDTA treatment and compared with the plain Col/HA gel. Strain sweeps were performed from 0.01% to 50% at 0.3 Hz, followed by frequency sweeps from 0.01 Hz to 50 Hz at 5% strain .

4.2.8 Coculture characterization in 3D alveolar construct

ATP and Live/Dead assay

ATP assay was conducted on mAlgs loaded with A549 cells and co-culture systems of A549 and NHLFs after EDTA treatment. For this, 200 μ l of reagent was prepared with 100 μ l culture medium and 100 μ l of Cell-Titer Glo Luminescent Cell Viability Assay solution, incubated for 5 minutes at 400 rpm, and rested for 25 minutes before reading luminescence values at the spectrophotometer. The protocol was slightly adapted in case of mAlg live/dead assay, when a fixed volume was withdrawn from mAlg suspension and diluted with the same volume of CellTiter-Glo® 2.0 in an eppendorf tube. Then, after the incubation time, the tube was centrifuged to collect the supernatant used for evaluation of luminescence. All conditions were performed with $n \geq 6$.

To evaluate cell viability post-EDTA treatment, a live/dead assay was performed on 3D constructs using a staining reagent. For 250 μl of reagent, a mixture of 125 μl culture medium and 125 μl PBS was prepared, adding 0.5 μl of calcein and 2 μl of ethidium homodimer. Samples were incubated for 30 minutes for dye penetration. Imaging was then performed by macro-confocal microscopy (Nikon AXR) and composite images were created with ethidium bromide red fluorescence representing dead nuclei and calcein AM green fluorescence representing live cells.

Inflammation of 3D alveolar constructs through PMA treatment

For treating 3D alveolar constructs, Phorbol 12-myristate 13-acetate (PMA) was applied at 10 nM and incubated for 24 hours, with daily medium changes to maintain optimal conditions.

ELISA kits and immunohistochemical staining

Cytokine analysis of the culture supernatant from 3D systems was conducted using a one-step ELISA kit (Bioscience) to quantify inflammatory responses after 0, 1, 4, and 7 days for human IL-6, IL-8, IL-1 β , and TNF- α . For alveolar sac formation, fixation was performed after 1, 4, and 7 days of culture for untreated and PMA-treated samples using 4% PFA. After three washes in PBS, the first immunostaining was performed, aiming at focusing on the pore formation, marking Surfactant protein-C (SP-C) secreted by A549, Zonula Occludens-1 (ZO-1) for tight junctions, and counterstaining via phalloidin (F-actin) and DAPI (for nuclear staining). Briefly the applied protocol was:

- PERMEABILIZATION: 0.1% TrytonX-100 (200-250 μl per each construct 10 min, RT);
- 3x PBS washes (5 mins each);
- BLOCKING: 1% BSA (i.e. 10 mg/ml, 200-250 μl per organoid 1 hr, RT);
- PRIMARY anti-SP-C (Abcam): 1 $\mu\text{g}/\text{ml}$ in Cyto-Q Immunodiluent (200-250 μl per construct overnight, 4°C, Parafilm and foil);
- 3x PBS washes (5 mins each);
- SECONDARY anti-rabbit-594 (Invitrogen): 1:1000 dilution in Cyto-Q Immunodiluent

(Bioscience, 200-250 μ l per construct, 1hr, RT);

- 3x PBS washes (5 mins each);
- CONJUGATED anti-ZO-1(488) (Invitrogen): 5 μ g/ml in Cyto-Q Immunodiluent (200-250 μ l per organoid 3 hrs, RT, Aluminium foil);
- 3x PBS washes (5 mins each);
- COUNTERSTAINING: Phalloidin-647 (Invitrogen, 1:400 in PBS) and DAPI (2 drops per ml of PBS) staining: 30 min RT.

Moreover, another set of fixed samples were stained to assess co-localization of cell populations and NHLF activation post-treatment. The samples were stained for Vimentin, a marker for NHLFs activation [20, 21], Cytokeratin 7 (CK-7, a marker for epithelial cells phenotype) and counterstained via phalloidin (F-actin) and DAPI (for nuclear staining). Briefly the applied protocol was:

- PERMEABILIZATION: 0.1% TrytonX-100 (200-250 μ l per each construct 10 min, RT);
- 3x PBS washes (5 mins each);
- BLOCKING: 1% BSA (i.e. 10 mg/ml, 200-250 μ l per organoid 1 hr, RT);
- PRIMARY anti-Vimentin: 1:250 in Cyto-Q Immunodiluent (200-250 μ l per construct overnight, 4°C, Parafilm and foil);
- 3x PBS washes (5 mins each);
- SECONDARY anti-rabbit-594 (Invitrogen): 1:1000 dilution in Cyto-Q Immunodiluent (Bioscience, 200-250 μ l per construct, 1hr, RT);
- 3x PBS washes (5 mins each);
- CONJUGATED anti-CK-7(488) (Abcam): 1:50 in Cyto-Q Immunodiluent (200-250 μ l per organoid 3 hrs, RT, Aluminium foil);
- 3x PBS washes (5 mins each);
- COUNTERSTAINING: Phalloidin-647 (Invitrogen, 1:400 in PBS) and DAPI (2 drops per ml of PBS) staining: 30 min RT.

Histological staining

PMA-treated and untreated 3D alveolar constructs were fixed, processed and embedded in paraffin, after which 6 mm sections were cut using a microtome (Leica Microsystems Inc., Buffalo Grove, IL) and mounted to slides. Slides were baked on a slide warmer for 1 h at 55 °C, followed by deparaffinization and rehydration. Then, Alcian blue staining was performed to identify GAGs deposits. Briefly, slides were stained in Alcian Blue solution (1% in 3% acetic acid, pH 2.5) for 30 mins. Then, slides were washed in running tap water for 2 minutes and rinse in distilled water. After that, the slides were dehydrated through 95% alcohol and 2 changes of absolute alcohol, 3 minutes each. Eventually a quick wash in xylene, before mounting the slide with resinous mounting medium.

Additionally, Picrosirius red staining was performed to highlight collagen remodeling in response to PMA treatment by means of Picrosirius red staining kit (Abcam), according to manufacturer protocol.

4.3 Results and discussion

4.3.1 mAlgs synthesis and characterization

The synthetic route that was chosen in order to obtain mAlgs is a bulk method based on emulsion with an external gelation process. Such methodology relies on the progressive addition of the water phase (i.e. Alg solution) in the spinning oil phase (i.e. Rapeseed oil and Span80 surfactant).

The constant high intensity stirring causes the breakup of the water phase into small droplets following the reaching of a *critical Capillary number* that depends on the viscosity ratio $p = \frac{\eta_D}{\eta_C}$ between dispersed and continuous phase [22]. For this reason, stationary rheology was used as a tool to get preliminary information about the expected behavior of the emulsion (Figure 4.4 and Figure 4.11).

In particular, flow curves were performed in order to preliminarily optimize the synthesis in terms of final droplet size prediction and feasibility of the whole procedure. Results showed a zero shear viscosity of the dispersed phase that is about 40 times or 120 times the one of the continuous phase for the 2%w/v and the 3%w/v Alg solution respectively. The RO sample shows the same behavior of a Newtonian fluid. However, the *shear-thinning* nature of the Alg solution becomes helpful when in high *shear rates*, as the viscosities drop by roughly an order of magnitude to very similar values for both Alg solutions (see **Figure 4.4A**).

More specific information can be obtained by plotting the viscosity ratio p as a function of *shear rate* (**Figure 4.4B**). Now, since RO has been showing a Newtonian behavior (i.e. viscosity being independent from *shear rate*), the only change in p value depends on the specific dependency for the Alg solutions $p(\dot{\gamma}) = \frac{\eta_{Alg}(\dot{\gamma})}{\eta_{RO}}$. It can be seen how at high shear rates, the two different samples converge to a pretty similar p value, with the Alg 3%w/v being slightly greater: this results can help predict an average mAlg dimension after the synthesis higher for the Alg 3%w/v sample, even though probably not significant.

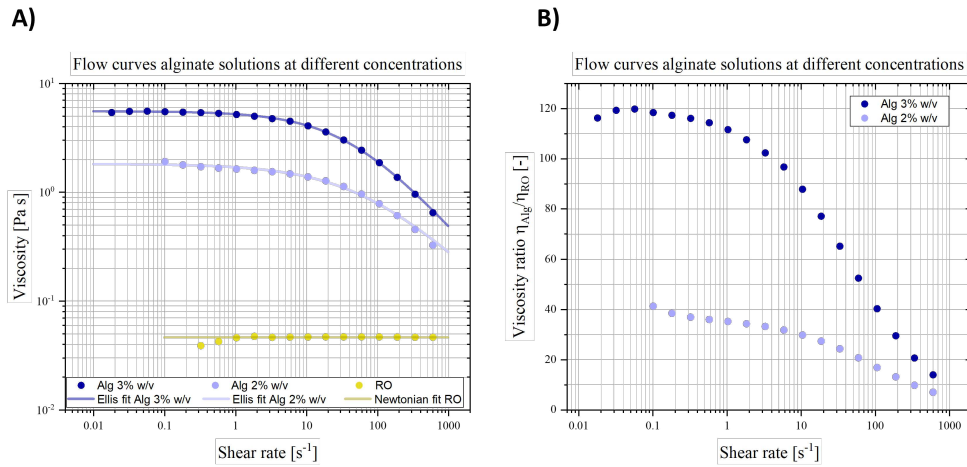


Figure 4.4: Rheological analysis of alginate precursors solutions at different concentrations (i.e. water phase) and rapeseed oil (i.e. oil phase of the emulsion). **(A)** Representative flow curves of 2% w/v and 3% w/v alginate solutions and rapeseed oil. Ellis fluid model was used to fit the data for alginate solutions, while the oil phase showed a newtonian behavior. **(B)** All viscosity data were normalized with respect to the oil phase viscosity, in order to provide the viscosity ratio index as a function of shear rate.

As a matter of fact, after the synthesis, purification and straining of mAlgs suspension, it was obtained a jelly-like solution, identical between samples and composed of densely packed mAlgs with a thin rim of water in between the microgels, as it can be seen in **Figure 4.5B** and **C**. That allowed to have a stable suspension, capable of flowing but still when a mechanical load was not applied, with a well-defined thixotropic behavior (see **Appendix D**).

When a quantitative dimensional analysis was run on the microgels, rheological predictions were confirmed, as the average diameter for 3% w/v mAlgs ($240 \pm 70 \mu\text{m}$) was a little higher than the 2% w/v mAlgs ($200 \pm 60 \mu\text{m}$), but not significantly. It was also possible to notice, both qualitatively from the optical microscopy images and quantitatively from the size distribution that the shape was homogeneous for both samples and size distributions were not excessively wide for a bulk microfabrication technique.

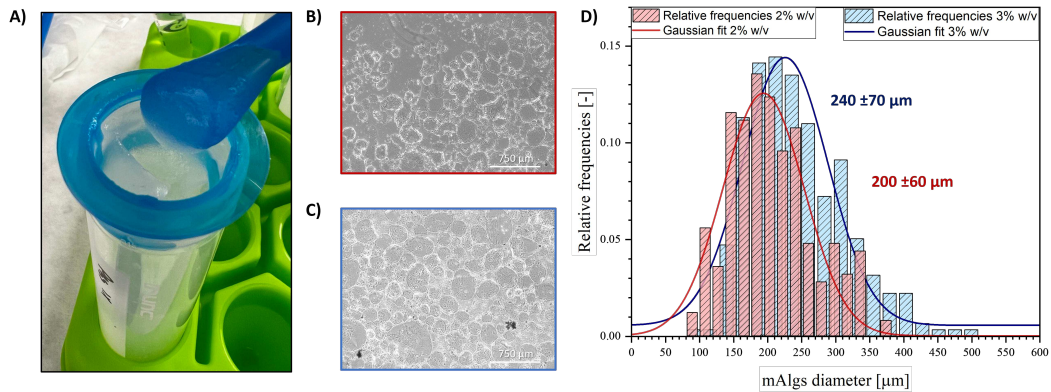


Figure 4.5: Representative image of mAlgs suspension obtained after the filtration and straining. It is visible the suspension's graininess, as it is composed of packed gelled microspheres (A) Representative optical microscopy with 4X magnification of mAlgs obtained from 2% w/v and 3% w/v alginate solution (B-C). Quantitative dimensional analysis of mAlgs' size obtained from 2% w/v and 3% w/v alginate solution. The histograms were obtained after the dimensional analysis and a Gaussian fit was performed to obtain the statistical parameters of the distribution, reported in terms of average size and standard deviation.(D)

Now, rheology has been providing an additional reason to chose the 2% w/v formulation to produce mAlgs, besides the smaller size and reduced standard deviation of dimensional distribution.

As a matter of fact, oscillatory rheology was performed on fully crosslinked 3D alginate pucks, capable of mimicking on a bigger scale the single mAlg's behavior. In particular, strain sweeps performed on 3D samples highlighted how the two different formulations not only show different storage (G') and loss (G'') moduli, but most interestingly a different critical strain value (γ_C in **Figure 4.6**) was observed.

The more concentrated Alg solution showed a smaller linear viscoelastic region (LVR), reaching a critical strain before non-linear deformation equal to 0.27%. On the other hand a less dense matrix, as the one obtained from 2% w/v Alg solution, allowed to reach a critical strain 1.5 times the previous one.

However, the most useful information for the experimental design has come from the oscillatory frequency sweep analysis. As a matter of fact, the typical response of a gel is highlighted (see **Appendix A**), with both G' and G'' moduli being parallel all along the frequency range. Furthermore, when the complex modulus $|G^*| = |G'| + |G''|$ is obtained from the frequency sweep data, the linear fit has been performed on the data points. This highlighted for both the samples the same trend, dependent on the ionic crosslinking of alginate [23]. Then, it allowed the identification of a low-frequency complex modulus $|G_0^*|$ used to identify the mesh size according to **Equation 4.1**.

In hydrogels, the concept of mesh size refers to the average distance between crosslinks in the polymer network, forming tiny pores or meshes throughout the material. These pores are small enough to retain water, giving the gel its shape, while also allowing certain molecules to move in or out depending on their size relative to the mesh. In this case, the 2% w/v alg gel has a mesh size equal to $\xi_{2\%} = 4.2 \text{ nm}$, while the other samples shows a mesh size equal to $\xi_{3\%} = 5.9 \text{ nm}$, indicating a mesh about 1.5 times denser in terms of crosslinks.

From this point of view, it is manifest how mesh size is a crucial factor because it directly affects the gel's properties, such as its mechanical strength, swelling behavior and, above all, permeability to different molecules [24]. For instance, a larger mesh size allows larger molecules to diffuse more easily, while a smaller mesh size restricts movement, enhancing stability but limiting transport.

In this context a higher mesh size is favorable, since it is needed to dissolve mAlgs by means of an EDTA solution. Now, since the treatment can be toxic or stressing for the encapsulated cells, the aim is to perform an EDTA treatment that is as effective as possible, acting in the smallest amount possible of time.

An approximation in order to model solute diffusion in hydrogel meshes is provided by Cuckier equation [25], that was later particoalized by Fujiyabu et al. [26, 27], according to which:

$$\frac{D}{D_0} = \exp\left(-\frac{r_s}{\xi}\right) \quad (4.2)$$

In which D is the solute diffusion coefficient in the hydrogel, D_0 is the diffusion coefficient of the solute in water, r_s is the solute radius and ξ is the hydrogel mesh size. Now, EDTA radius can be estimated. Therefore, it can be calculated the ratio between the EDTA molecule in the two different hydrogel meshes obtained from the 2% and 3% alginate solution precursor:

$$\frac{D_{3\%}}{D_{2\%}} = \frac{\exp\left(-\frac{r_{EDTA}}{\xi_{3\%}}\right)}{\exp\left(-\frac{r_{EDTA}}{\xi_{2\%}}\right)} \sim 0.9 \quad (4.3)$$

For this reason, it is possible to suppose that the choice of the 2% w/v Alg solution is the one that provides the better results, since:

- It provides a less viscous solution, easier to handle in the first steps;
- The obtained mAlgs show a slightly more uniform size distribution;
- The obtained gel has a larger mesh size and this allows a penetration of EDTA molecule that is around 10% more efficient.

4.3.2 mAlgs seeding density optimization

Now, after selecting the best alginate concentration for mAlgs, it was investigated the formation of the alveolar architecture in the Col/HA gel. In order to do so, different seeding densities were tested in terms of volume ratios between r-mAlgs suspension and Col/HA-488 solution¹⁰.

In particular, it was investigated also the effect of EDTA treatment. The confocal microscopy was implemented to monitor the formation of porosity in the constructs (**Figure 4.7**). So, the 3:5 ratio was identified as the best seeding density: the reason for that double. Firstly, it helped the formation of an adequate layer of gel -that will be seeded with NHLFs in following experiments- mimicking the ECM-like septum between pores in the alveoli.

Then, after the EDTA treatment, this specific mAlgs density leaves behind a fully porous substrate with enough inter-connectivity between pores, but still a freestanding structure that does not collapse during culture.

¹⁰This was possible due to the nature of mAlgs suspension, that can be fairly easily suspended and pipetted.

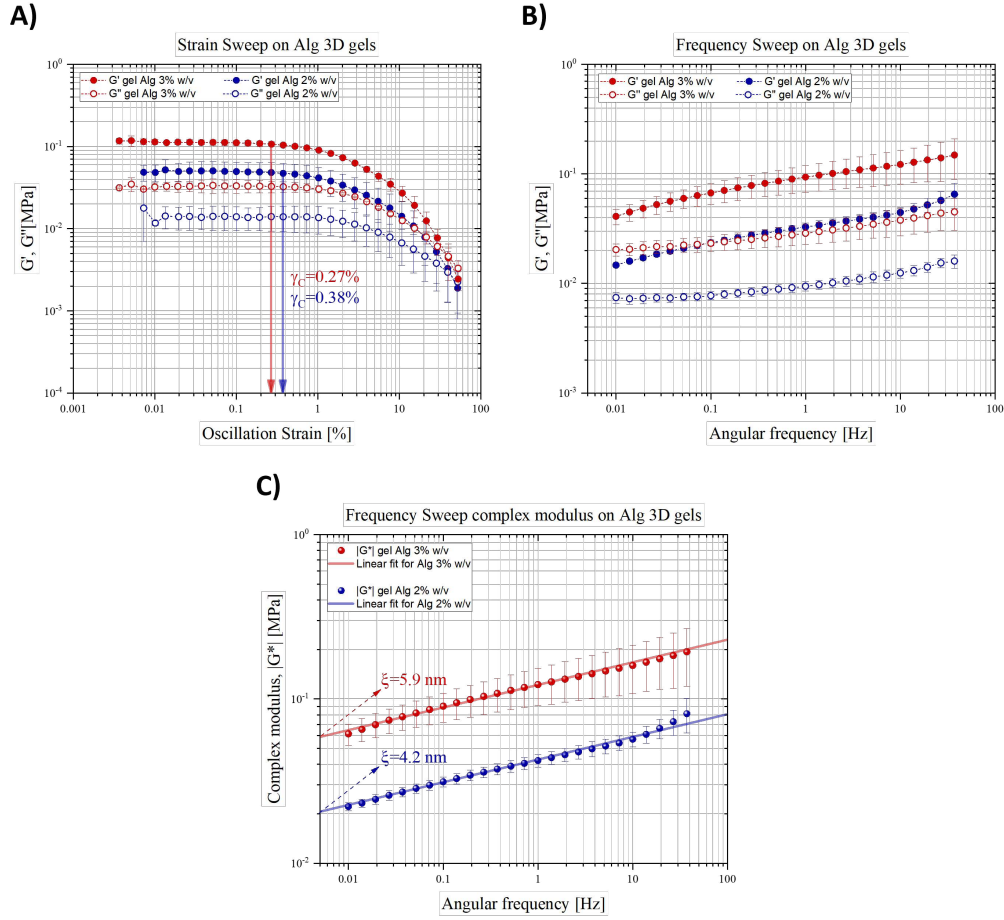


Figure 4.6: Oscillatory rheological analysis of alginate 3D pucks starting from Alg solutions at different concentrations at T=37°C. **(A)** Strain sweep analysis on 3D Alg pucks. Critical strain values for each sample are reported as γ_C , in order to highlight the linear viscoelastic region (LVR). **(B)** Frequency sweep analysis run in the LVR region on 3D Alg pucks. Both storage (G') and loss (G'') moduli are reported as a function of oscillatory frequency. **(C)** Complex moduli as a function of oscillatory frequency obtained from frequency sweep data. Linear fits are reported and from the intercept values the meshsize ξ was calculated and reported for each sample.

Differently, lower seeding densities risk creating structures with a certain degree of disproportion between septa and pores, in a conformation that does not allow for good pore interconnectivity, leading to less efficient dissolution of mAlgs.

In contrast, seeding of mAlgs at densities greater than that selected, while maintaining the excellent efficiency of the mAlgs dissolution procedure, nevertheless this does not leave a structure capable of sustaining itself. In fact, the resulting final 3D alveolar structure loses its spherical shape prior to treatment. In case of the highest mAlgs concentration (5:3 ratio), the residual red signal after EDTA treatment is possibly due to the synthetic methodology of r-mAlg. The presence of red fluorescent micro-beads in mAlgs after dissolution are still left in the system and might diffuse in the Col/HA gel, residing there still after the culture.

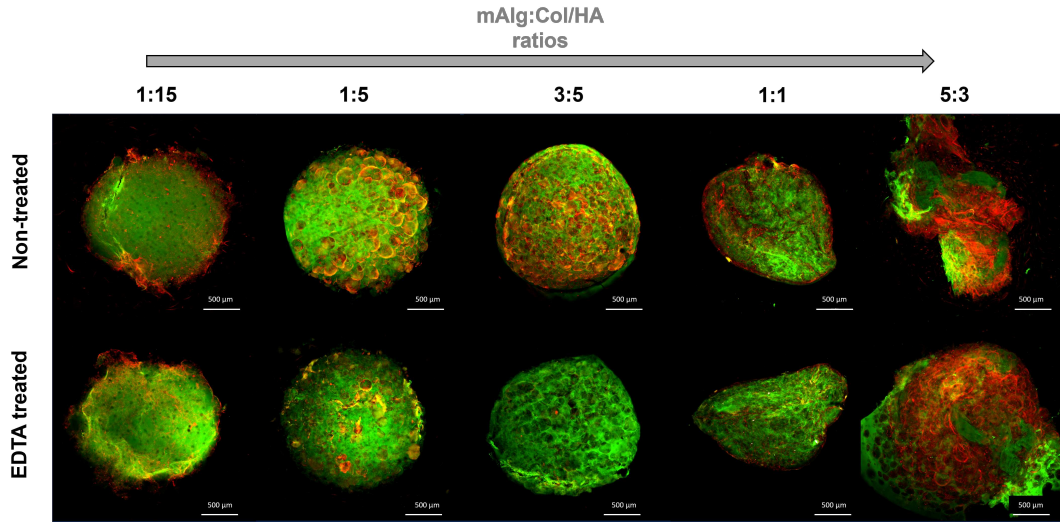


Figure 4.7: Schematic representation of the mAlgs seeding optimization procedure by confocal microscopy investigation. From left to right, increasing loading ratios of r-mAlgs into Alexa Fluor-488 labeled Col/HA gels. Results are reported before and after EDTA treatment, in order to visualize the structural support offered by the gelled matrix after the dissolution of the mAlgs. Scale bars: 500 μm

4.3.3 Effects of EDTA treatment: mechanical properties and NHLFs viability

Now, to this point both the 2% w/v alginate solution was selected to produce the mAlgs and also their seeding density (3:5 ratio) was selected to produce the better mimicking alveolar construct. The formation of the pores allowed a drastic change in mechanical properties (**Figure 4.8**).

Rheology was used as a main tool to infer about the variations in the structure, ideally comparing the behavior under oscillatory strain of the alveolar pucks. Briefly, strain sweeps strongly highlighted the effect of porosity on the alveolar sistem LVR. In particular, the 3D construct before the EDTA treatment showed a critical strain $\gamma_c = 10\%$, while after the EDTA treatment the novel porosity reduced about the half the strain range in which the construct holds together (**Figure 4.8 A**).

This is likely due to the presence of a hollow network in the system, in which stress is highly concentrated in the septa of the material, while the Col/HA + mAlg system before EDTA treatment offers a better possibility to stress distribution, still behaving as a composite material with a specific yield.

Frequency sweeps, instead, allowed to better observe the actual formation of the pores in the HA/Col 3D pucks after the treatment, as both the storage (G') and loss (G'') moduli decrease in the first two frequency decades of about $1/4$ (**Figure 4.8 B**).

It has been considered a full untreated Col/HA puck, in which the mechanical properties reflect those of the biomimetic gel that makes up the septa between alveoli. The comparison between this plain material and the mAlg loaded samples highlights how loading mAlgs in the construct increases slightly both G' and G'' , but after the EDTA treatment the presence of a porous structure lower the storage modulus to about 0.08 kPa (at 1 Hz). Another interesting difference can be noticed especially at high frequencies, where the porosity seems to introduce a high deviation in terms of behavior. At higher frequencies (starting from 4Hz for the untreated sample and from 1 Hz for the treated sample) not only storage modulus soars to higher values than in the plain Col/HA, but EDTA-treated and untreated samples reach very close values.

As a matter of fact, it has been widely investigated in literature the mechanical properties of lung tissue at the level of alveoli. More in detail, it was made clear the different mechanics that governing the fabric at different scales. For example, the hyperelastic nature of the alveolar walls [28] -which in our case is the Col/HA system- was observed, as well as the macromechanical behavior of the composite porous structure [29] that, on a larger scale, contains different pores -in our case the 3D alveolar system after treatment with EDTA.

4.3.4 NHLFs seeding density optimization and viability in time

With the previous set of experiments it was possible to optimize a fabrication method to obtain the 3D alveolar architecture typical of lung's alveoli. Now, the efforts have been focusing on the development of efficient seeding methodologies for both stromal and epithelial cells.

Once it was made clear the efficacy of the EDTA treatment, it was necessary to prove that once the NHLFs were preliminarily seeded in the Col/HA gel, the EDTA treatment wouldn't have a cytotoxic effect. To this aim, both a live/dead assay after the treatment and an ATP assay in time were run on the treated NHLFs-loaded 3D alveolar constructs (**Figure 4.9**).

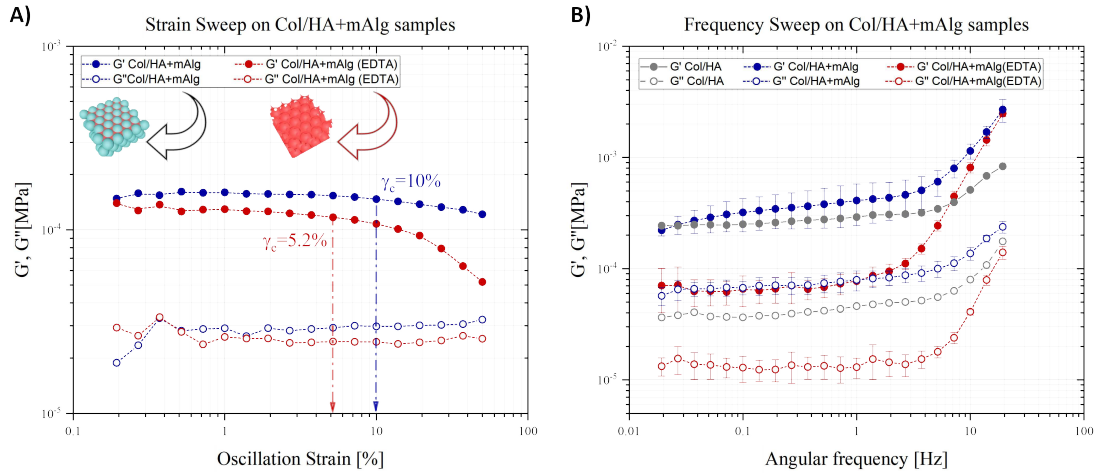


Figure 4.8: Oscillatory rheology performed on 3D substrate before and after EDTA treatment. Representative strain sweeps performed on mAlgs-loaded Col/HA gel puck before (in blue) and after (in red) EDTA treatment. Critical deformations γ_C are reported, marking for each sample the LVR (**A**). Frequency sweeps performed on mAlgs-loaded Col/HA gel puck before (in blue) and after (in red) EDTA treatment. Blank Col/HA gel puck are used as a mechanical control and as a model of the mechanical behavior of the septum (in grey)(**B**).

Briefly, the live/dead assay showed far more green staining of the cell population than red staining for both the CTR and the EDTA-treated construct. In particular, it can be seen that likely the few dead cells in the CTR construct are indeed the remaining cells obtained from the culture pellet after trypsinization. However, no difference in terms of cytotoxic effect on the cells consequent to EDTA treatment can be observed (**Figure 4.9A**) [30].

What is more, the ATP assay further confirmed quantitatively that the cells that can be observed after treatment are totally able to proliferate and sustain a culture for days, since the viability has been observed to progressively increase after 3, 7 and 14 days (**Figure 4.9B**).

Then, the inspection by confocal microscopy of the CSFE-labelled NHLFs loaded in Col/HA gels shown in **Figure 4.10** allowed to observe the optimized seeding density (8×10^6 cells/ml), as well as the proliferation of stromal cells. Before the EDTA treatment it was observed that the presence of r-mAlgs was able to support the overall structure.

However, when compared with EDTA-treated constructs, the proliferation of cells following treatment can again be observed after 4, 7 and 14 days. What is more, once the mAlgs are dissolved, it can be observed that the NHLFs fail to support the alveolar structure, leading to the collapse of some pores as a result of their progressive squeezing.

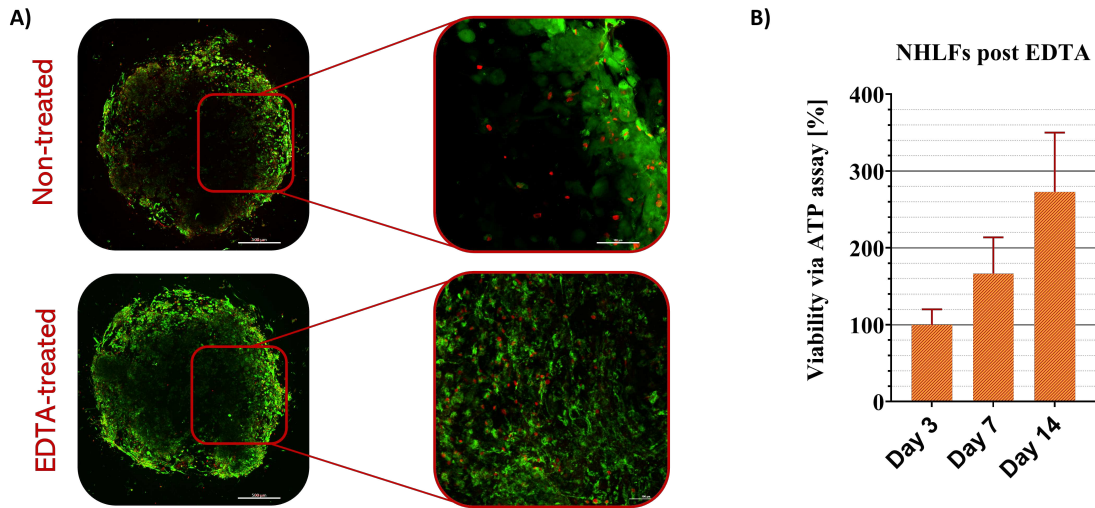


Figure 4.9: Effects of EDTA treatment on pre-embedded NHLFs in Col/HA gels. **Live/dead** assay performed on $5\mu\text{l}$ constructs with NHLFs seeded in the Col/HA gels before and after the 1hr treatment with EDTA 0.01M. Images acquired by confocal microscopy using 4X and 10X magnification (**A**). NHLFs viability in time (after 3, 7 and 14 days of culture) by ATP luminescence assay after the EDTA treatment. (**B**)

And on top of that, the shape of the 3D construct as a whole also tends to collapse, losing sphericity-as observed from 4X magnification. These observations on the successful seeding of stromal cells in a porous 3D matrix underscored the need for an additional cell population to provide structural solidity, guiding the next fabrication step.

4.3.5 A549 encapsulation in mAlgs

The synthetic route had to be redesigned in order to obtain mAlgs loaded with A549 cells by the same bulk method based on emulsion with an external gelation process. This new synthetic route involves from the beginning an alginate solution, sterilized by autoclaving in which the A549 cells were suspended at a 20×10^6 cells/ml seeding density.

In particular, flow curves showed a zero shear viscosity of the sterile Alg solution that is about 1/10 the one of non-autoclaved 2%w/v Alg solution. Now, the *shear-thinning* nature of the Alg solution changes after the thermal treatment, as the autoclaved solution clearly shows a drop-off at higher in high *shear rates*, as the viscosities drop by roughly an order of magnitude to very similar values for both Alg solutions (see **Figure 4.11A**).

And when viscosity ratios were obtained by normalization to the oil phase viscosity, it can be observed how the sterilization process lowers the viscosity ratio to $p \sim 5$ at 1000s^{-1}

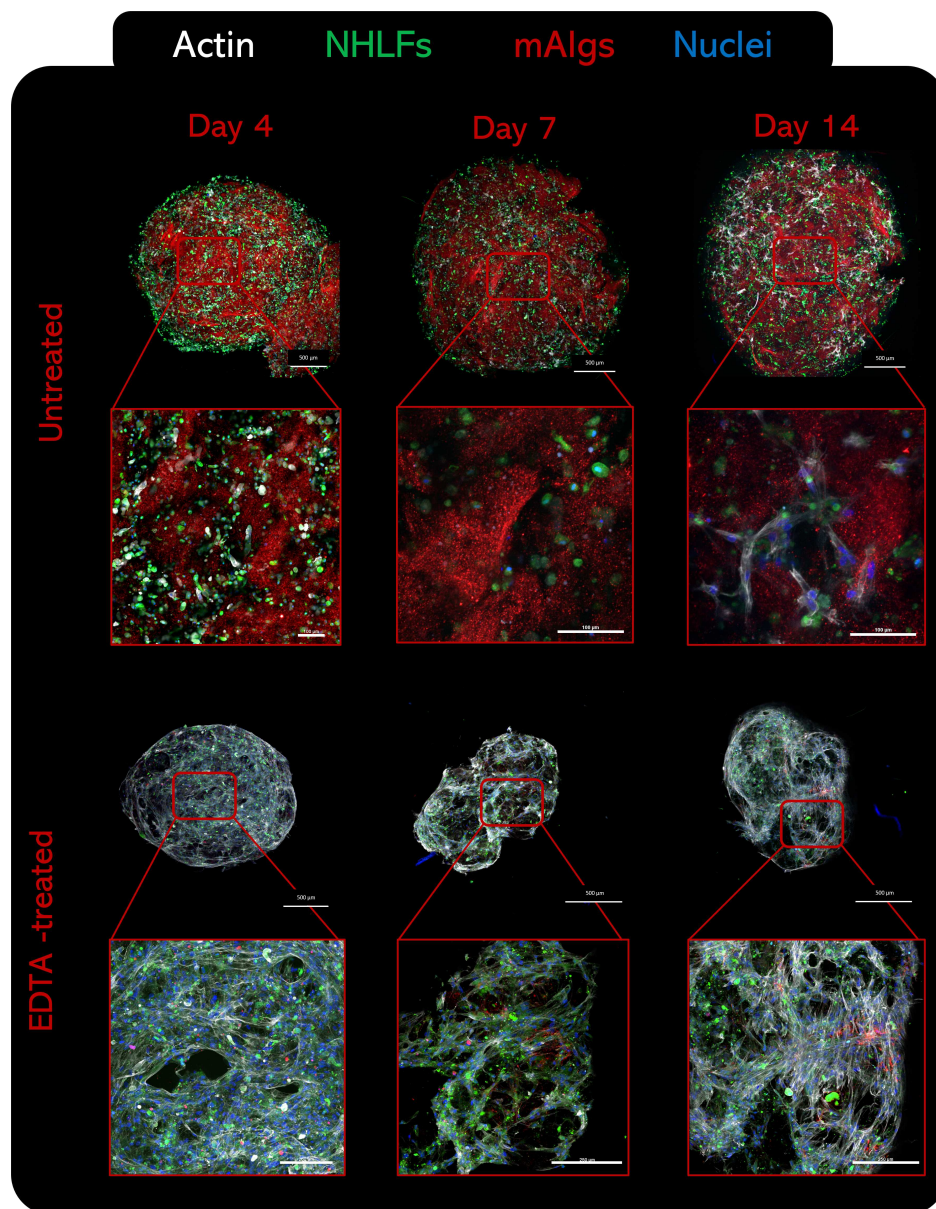


Figure 4.10: Schematic representation of the NHLFs seeding optimization procedure by confocal microscopy investigation, using 4X and 10 X magnification. CSFE-labeled NHLFs were seeded in the Col/HA (8×10^6 cells/ml) together with r-mAlgs and treated with EDTA. Following the 14 days culture, NHLFs proliferated, but also progressively caused the collapse of structures over time, affecting porosity. This highlighted the need for further structural support after the mAlgs dissolution. Scale bars: $500\mu\text{m}$ for 4X magnification and $200\mu\text{m}$ for 10X magnification. In red, the fluorescent r-mAlgs, in green the CSFE-stained NHLFs, counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue).

(**Figure 4.11B**), which is the lowest value reached also when compared to the data in **Figure 4.4B**. For this reason it was also possible to predict a different outcome in the dimensional distribution following gelation.

And indeed the dimensional analysis conducted on these newly synthesized mAlgs returned a result predicted by the flow curves. In fact, analysis of the diameters of the mAlgs

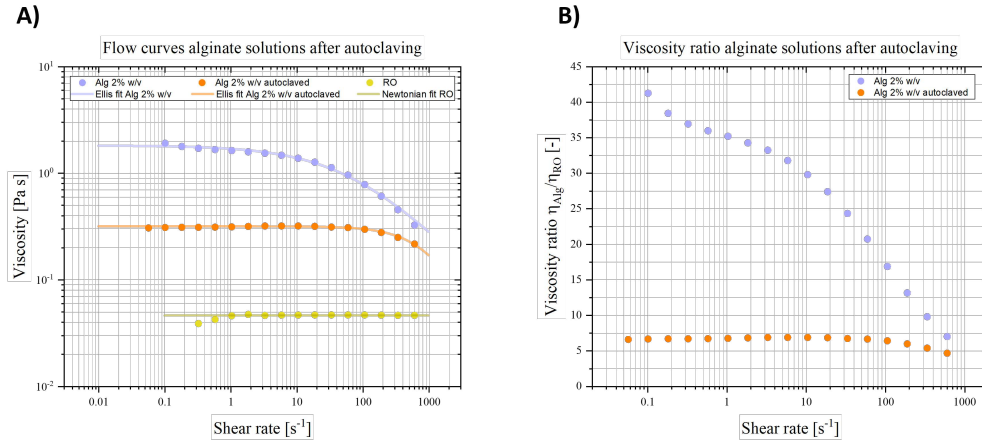


Figure 4.11: Rheological analysis of alginate precursor solutions before and after autoclaving (i.e. water phase) and rapeseed oil (i.e. oil phase of the emulsion). **(A)** Representative flow curves of 2% w/v alginate solution before and after autoclave sterilization and rapeseed oil. Ellis fluid model was used to fit the data for alginate solutions, while the oil phase showed a Newtonian behavior. **(B)** All viscosity data were normalized with respect to the oil phase viscosity, in order to provide the viscosity ratio index as a function of shear rate.

with the A549 encapsulated within the microgels shows that not only is the mean diameter reduced compared with the white mAlgs (from 206 to 150 or 750 μm), but that the standard deviation does not vary more.

What is more, comparing the seeded mAlgs with the two different cell densities of A549 we derive that the effect of loading is not manifested significantly, but nevertheless it seems to bring an increase in diameter, but also an increase in standard deviation when going from 10 to 20×10^6 cells/ml (**Figure 4.2B**).

Instead, focusing more on the actual encapsulation of epithelial cells, **Figure 4.2A** shows the successful encapsulation of the PKH26-tagged A549 cells in the mAlgs. However, the problem of optimization to obtain a viable A549 encapsulation procedure in a mechanically stable gel is not trivial and is described in **Appendix E**. By the end of this optimization process, the mAlgs behaved as a stable, biocompatible carrier for the encapsulated cells, as they were also able to proliferate in time, showing and increasing ATP signal in time when cultured up to 10 days. Such quality has also an experimental practice quality, as it enables to split the production of the final construct in different experimental sessions, making the overall synthetic process more easily addressed.

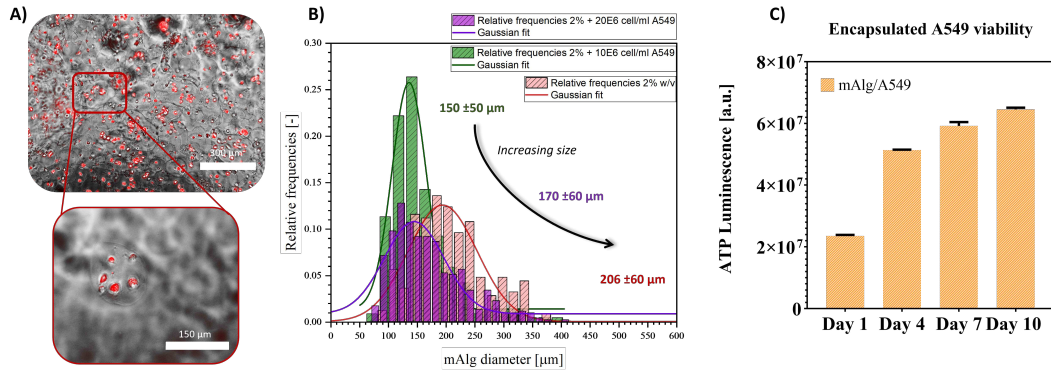


Figure 4.12: Process of A549 encapsulation in mAlgs. Representative images of PKH26-labeled A549 after the encapsulation in mAlgs, image by fluorescent microscope. Scale bars: 300 μm and 150 μm for 10X and 20X magnifications respectively (A). Quantitative dimensional analysis of blank and A549-loaded mAlgs' size obtained from 2% w/v alginate solution, at two different encapsulation cell densities: 10×10^6 and 20×10^6 cells/ml. The histograms were obtained after the dimensional analysis and a Gaussian fit was performed to obtain the statistical parameters of the distribution, reported in terms of average size and standard deviation (B). A549 cells viability after encapsulation via ATP luminescence assay as a function of days of suspension in culture media (Day 1, 4, 7 and 10) (C).

4.3.6 3D coculture system optimization

Now, up to this part of the work a 3D alveolar construct was successfully fabricated with a specific architecture resembling the one of human alveoli. Then, it was addressed the seeding of a stromal population of cells in the construct and the optimization of a biocompatible fabrication technique.

The steps that follow instead focus on the introduction of an epithelial cell population (i.e. A549 cell line) in order to form a 3D coculture system. Moreover, in order to produce a fully biomimetic construct an essential concept has been taken into consideration: the specific colocalization of different cell types.

As a matter of fact, it is not only necessary that the epithelial cells are seeded in the system, but they need to reside in a specific location, covering the pores' walls, just like in human alveoli. In the actual alveolar sacs the stromal component of cells' population resides in the ECM [31], while the inner layer of alveolar walls are covered by an epithelial barrier [32].

Same way, to this moment, NHLFs has been successfully seeded in the ECM-like gel and they are actually embedded in that matrix. At this point it is sought a seeding methodology for epithelial cells that would allow a specific seeding on the outer wall, avoiding as much as possible their penetration in the gel where stromal cells are embedded.

A549 seeding by conventional cells suspension

In traditional methodologies for seeding alveolar constructs, epithelial cells are typically introduced as a suspension, allowing them to settle and adhere passively to the substrate surface, with possibly some additional flow induced by plate tilting. This approach relies heavily on the natural adhesion properties of epithelial cells to anchor effectively within the construct over a specific period [14].

For instance, in preliminary studies on our Col/HA constructs, we observed that after 24 hours in dynamic culture, several cells had adhered to the scaffold, showing early evidence of monolayer formation. Although widely used, this technique can result in uneven cell distribution and requires prolonged adhesion times, particularly in constructs with intricate pore architectures or high porosity, where initial cell loss may be significant.

As a matter of fact, from **Figure 4.13** it can be seen the effects of this specific seeding procedure. In particular, z-stacks maximum projections are reported, where A549 cells are labelled in red with PKH26 stain and in green NHLFs cells labelled with CFSE in a system cultured up to 14 days. What seems evident the uneven distribution of the A549 cells on the outer rim of the construct, even though a dynamic culture was applied. Besides, it also shows how the actual cell density in the construct is not constant, as a gradient of A549 concentration exists due to their diffusion in the construct.

In particular, it can be seen from the z-slices of the red channels in **Figure 4.14**, as the A549 struggle to penetrate the three-dimensional construct. As a matter of fact, the construct has a specific characteristic, since it is endowed with an internal porosity, but an outer layer of ECM-like gel, just like an human alveolus possessing interstitial fibroblast playing a role in septal elastogenesis [33].

This means that the A549 seeded that way are supposed to migrate into this first layer, to have the ability then to access the inner part of the construct. However this means that the cells cannot penetrate directly into the scaffold, but have to diffuse through the matrix. This issue brings two main problems, faced also in previous works: the slow diffusion of cells through the matrix and their mixing with the stromal cells.

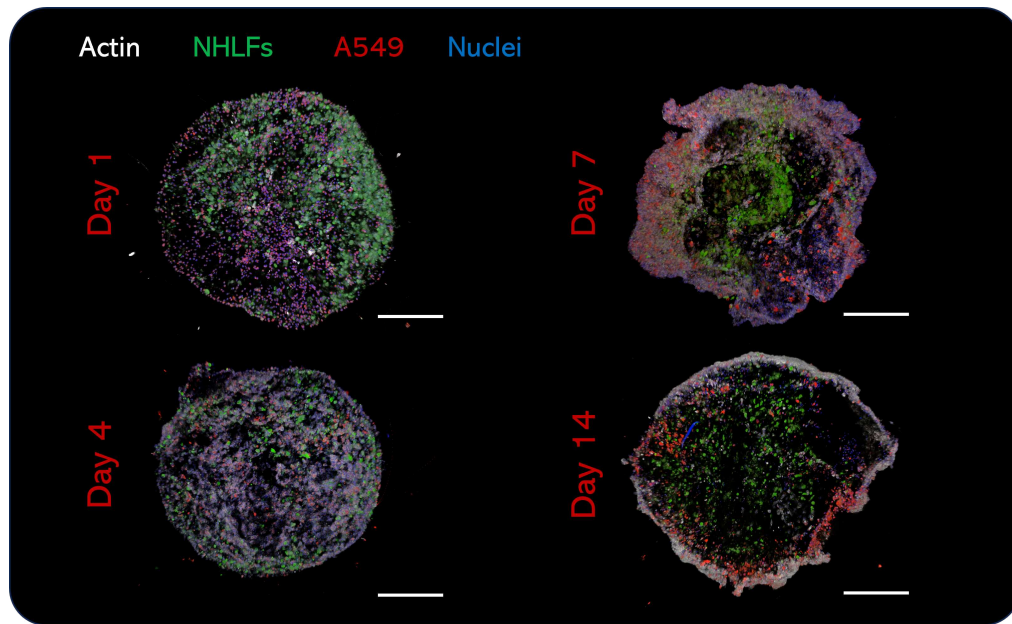


Figure 4.13: Representative maximum projection images of confocal microscopy acquisition performed on a CSFE-labeled NHLFs- loaded 3D alveolar tissue constructs left overnight in a PKH26-labeled A549 suspension overnight and cultured for 1, 4, 7 and 14 days to obtain a coculture system, in order to study the A549 diffusion in the $5\mu\text{l}$ constructs. In red, the PKH26-labeled A549, in green the CSFE-stained NHLFs; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: $500\mu\text{m}$

Indeed, after 14 days of culture the system is still showing very few cells that were able to penetrate in the inner part of the construct, while most of them were still residing on the outer rim of the construct. What was seen is that after 7 days they were able to penetrate about $220 \pm 80\mu\text{m}$ in the construct and after 14 days $400 \pm 150\mu\text{m}$. Moreover, those that penetrated were mixed with NHLFs in the septum, and quite rarely populating the pore walls.

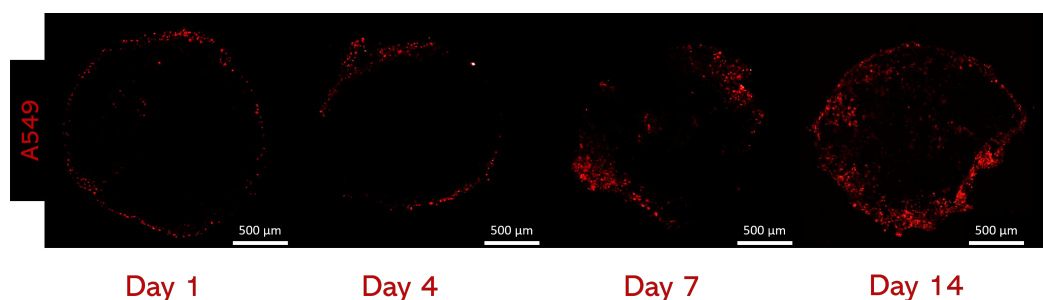


Figure 4.14: Representative Snaps from z-stack acquisition by confocal microscopy (4X magnification) of PKH26-labelled A549 penetration in NHLFs- loaded 3D alveolar systems. The acquisitions have been performed after 1, 4, 7 and 14 days of incubation of the $5\mu\text{l}$ constructs with the fluorescently tagged A549 suspension (40×10^6 cells/ml or equivalently 2×10^5 cells/construct). From this image is possible to notice the struggle A549 make to penetrate the inner parts of the construct, as well as their concentration gradient. Scale bars: $500\mu\text{m}$

These issues became quite evident as this seeding procedure was performed on 3D macroscopic pucks. As a matter of fact, the low diffusion becomes even more problematic, since the diffusion path is orders of magnitude larger: this means that the cells need more than 14 days to populate the construct -after 7 days there are mainly fibroblasts as in **Figure 4.15**- and A549's concentration gradients become even more evident.

Furthermore, as it can be seen from the following confocal acquisition how the epithelial cells are populating the hydrogel, rather than being present only on the outer rim of the pores. This characteristic represents a great limit of this current seeding method for alveolar structures. As a matter of fact, when dealing with human alveoli, on the epithelial surface there are the ATI and ATII cells, while mesenchymal cells are shown within the alveolar interstitium, in particular fibroblasts and myofibroblasts [34].

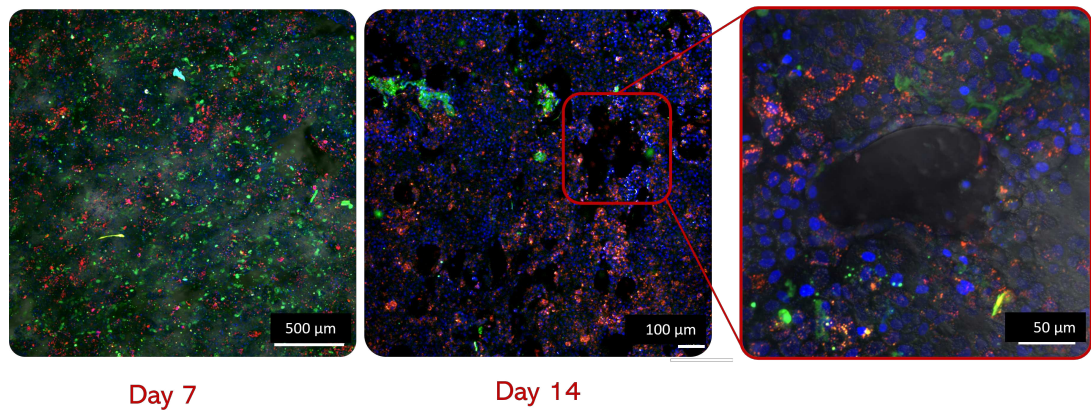


Figure 4.15: Representative maximum projection image of confocal microscopy acquisition (4X and 20X magnifications) performed on a CSFE-labeled NHLFs- loaded 3D alveolar puck left overnight in a PKH26-labeled A549 suspension overnight and cultured for 14 days to obtain a coculture system. In red, the PKH26-labeled A549, in green the CSFE-stained NHLFs; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: 500 μm and 100 μm for 4X magnifications and 50 μm for 20X magnification.

A549 seeding by A549/mAlgs dissolution

Therefore, in the framework of this fabrication route it was proposed a novel approach to the problem of a proper epithelialization, by means of the encapsulation procedure applied to the A549 in mAlgs. As a matter of fact, the idea was to have the epithelial cells safely delivered *a priori* into the pores, and only after the EDTA treatment let them attach to the pore's walls, without the need for them to migrate.

More in detail, once the EDTA treatment was performed, the 3D systems needed just 4 days to show a complete pore walls epithelialization (**Figure 4.16**). However, the systems were able to support the growth for even more than 4 days-as it will be seen also in **subsection 4.3.7**. Actually, ATP assay performed on the coculture system proved the fact that the system is fully capable of sustaining the culture also for 14 days. However, there is no need for such long culture time, since 4 days are sufficient to consider the 3D construct mature enough for further testings: this represents a main advantage compared to the usual cells suspension seeding procedure in terms of experimental times.

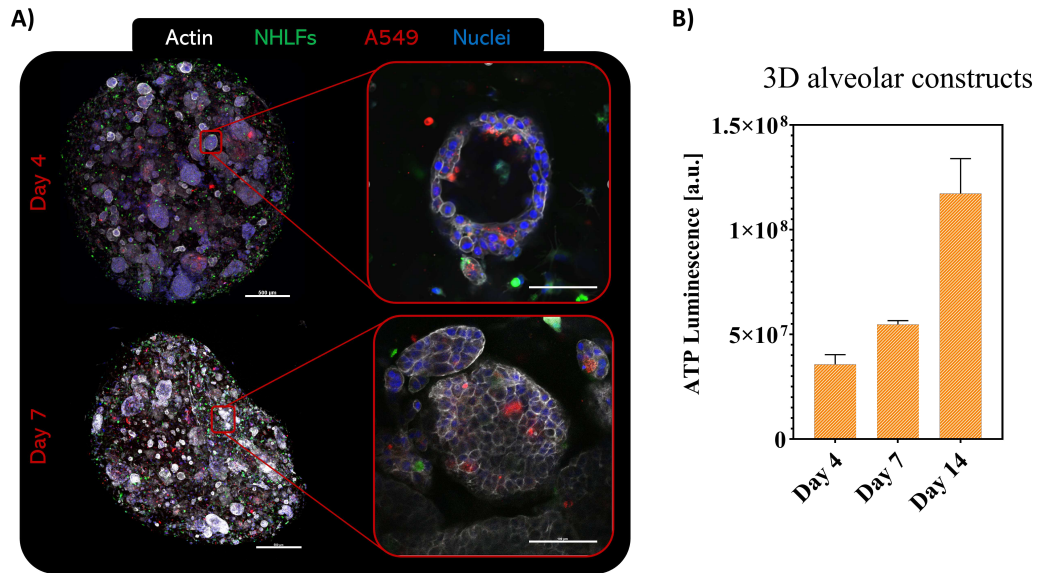


Figure 4.16: Representative images of confocal microscopy acquisition (4X and 10X magnification) performed on a CSFE-labeled NHLFs- loaded 3D alveolar tissue constructs in which the PKH26-labeled A549 were seeded in mAlgs and then treated with EDTA solution and cultured for 4 and 7 days. In red, the PKH26-labeled A549, in green the CSFE-stained NHLFs; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: $500\mu\text{m}$ for 4X magnification and $100\mu\text{m}$ for 10X magnification.

4.3.7 Inflammatory insult by PMA treatment: effect on alveolar walls formation

Once optimized the epithelial cells seeding procedure, now the final 3D alveolar system was complete. Therefore, the first characterization that needed to be done was the actual formation of a functional alveolar epithelial cells barrier. For this reason the system was immunostained using specific markers: ZO-1, SPC, with counterstaining for actin filaments and nuclei (DAPI).

ZO-1 is typically used to visualize tight junctions, indicating cell-cell adhesion and epithelial barrier integrity, while SPC (surfactant protein C) serves as a marker for alveolar epithelial type II (ATII) cells, reflecting functional properties of alveolar epithelial cells. The actin stain delineates the cytoskeleton and aids in understanding the cell organization within the matrix, while DAPI highlights cell nuclei, allowing for overall cell distribution and density assessment (4.174.18).

Over 14 days of culture, the immunostaining revealed that A549 epithelial cells did not penetrate deeper into the ECM, remaining primarily confined at the surface or peripheral layers of the pores. This spatial restriction could be attributed to either the cell-cell adhesion maintained by ZO-1 interactions or potentially to the structural density of the ECM itself, which might prevent deeper epithelial infiltration, ensuring that the fibroblasts within the matrix maintain distinct stromal regions.

Additionally, upon stimulation with PMA, an inflammatory agent, the construct showed no substantial alterations in ZO-1, SPC, or actin expression patterns, suggesting that the inflammatory conditions did not notably disrupt the epithelial structure. This stability in marker expression implies that the construct retains its structural and cellular organization under inflammatory stress, offering for sure a valuable insight into its potential resilience in modeling chronic lung pathologies.

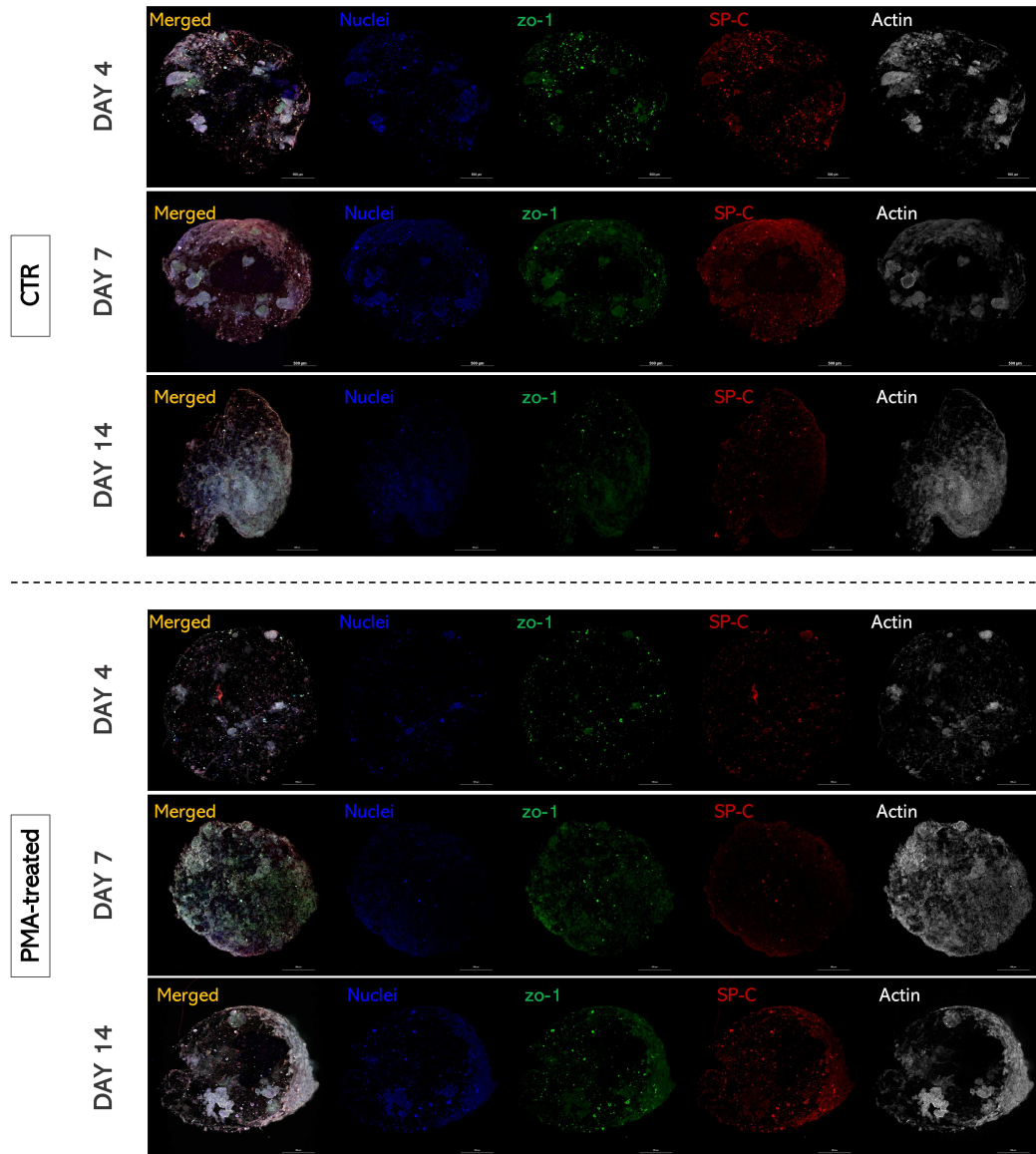


Figure 4.17: Representative immunostaining images of 4X confocal microscopy acquisition on 3D alveolar tissue constructs after 4, 7 and 14 days from PMA treatment (untreated samples acting as CTR). In red, the SP-C expressed by A549, in green the ZO-1 to mark tight junctions formation; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: 500 μ m.

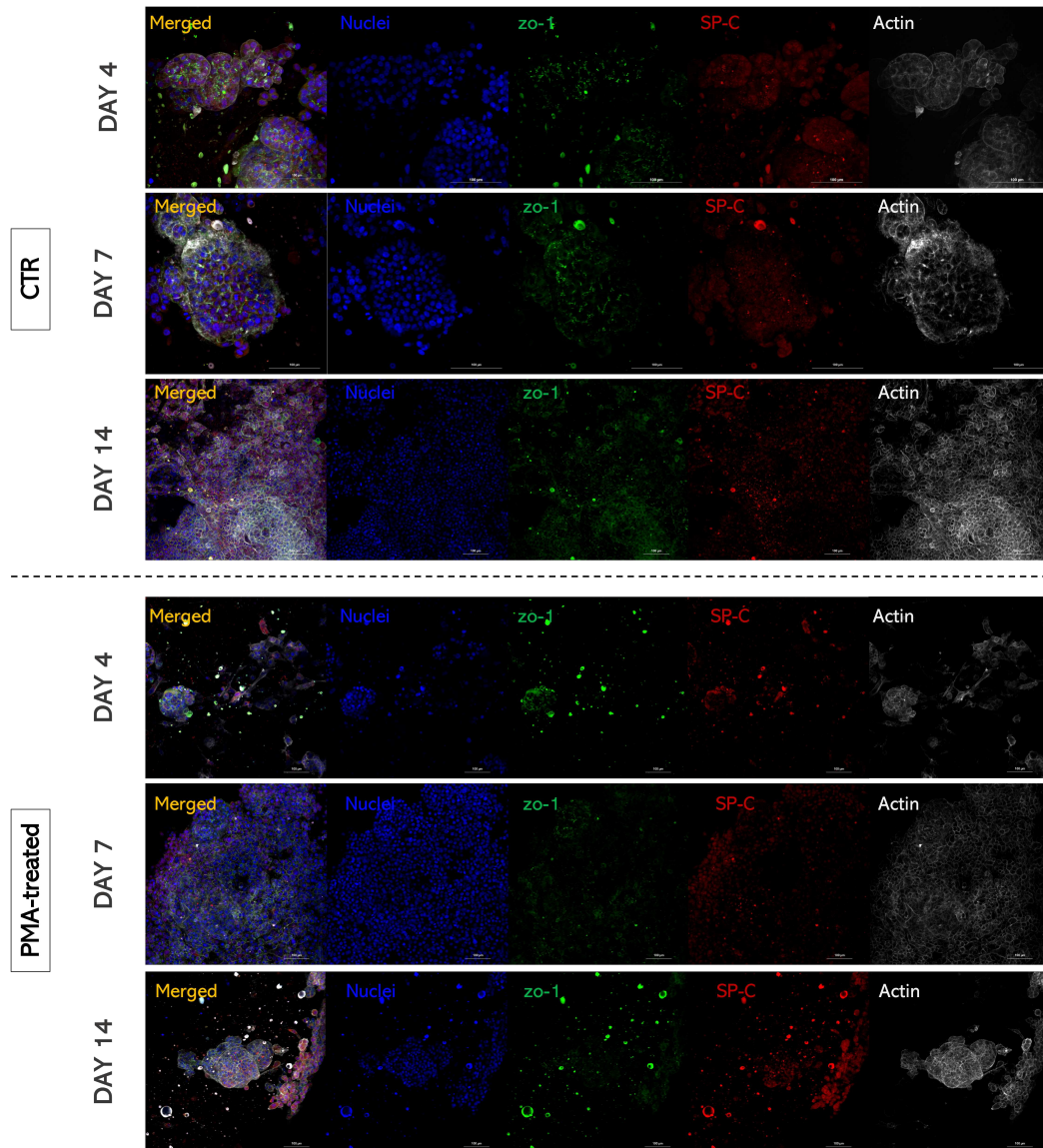


Figure 4.18: Representative immunostaining images of 10X confocal microscopy acquisition on 3D alveolar tissue constructs after 4, 7 and 14 days from PMA treatment (untreated samples acting as CTR). In red, the SP-C expressed by A549, in green the ZO-1 to mark tight junctions formation; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: $100\mu\text{m}$.

4.3.8 Inflammatory insult by PMA treatment: effect on cytokines expression, NHLFs activation and fibrotic response

Now, even though the PMA didn't show a clear effect on pore formations and barrier stability, it is known to have an effect on proinflammatory cytokines production. More in detail, PMA treatment led to a measurable effect on inflammatory cytokine production, particularly with increased IL-6 and IL-1 expression after 7 days, while IL-8 secretion was slightly elevated in control samples at both 4 and 7 days.

IL-6 is a key mediators in inflammatory signaling, commonly associated with acute phase responses and remodeling processes, especially in tissue subjected to external stressors or injury. Notably, IL-6 in particular plays a role in fibroblast activation and epithelial cell response, often associated with wound healing and fibrosis [35, 36]. Meanwhile, IL-8, primarily a neutrophil attractant, shows only modest increases in CTR, aligning with a limited inflammatory escalation within the construct and suggesting a nuanced cellular response to PMA.

Interestingly, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, both strong pro-inflammatory cytokines often elevated in response to intense inflammatory stimuli, did not show significant changes in this model. This suggests that, while PMA can modulate certain inflammatory pathways, it may not trigger the same exaggerated responses typically observed in traditional 2D models [37].

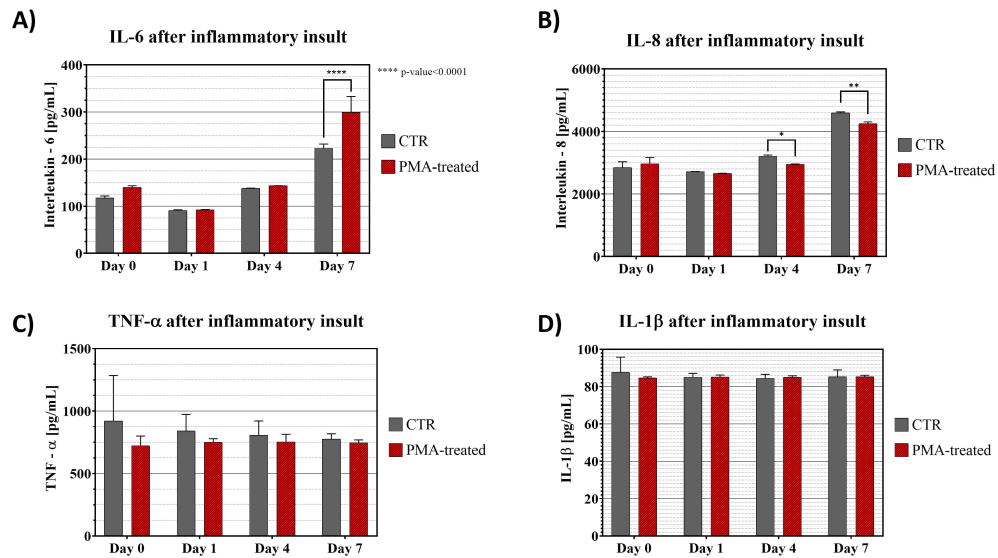


Figure 4.19: Inflammatory cytokines expression by quantitative ELISA kits after PMA insult on 3D Alveolar systems. IL-6, IL-8, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ cytokines expressions are reported as a function of days of culture. Day 0 represents the samples' supernatant after the 24 hrs treatment. $p\text{-value} < 0.0001$ was considered highly significant

This finding supports the hypothesis that the 3D construct could more closely emulate *in vivo* lung tissue responses, where overly high concentrations of PMA used in 2D cultures may fail to represent realistic *in vivo* conditions. This nuanced inflammatory response underlines the construct’s potential as a more physiologically accurate model, providing insight into the behavior of lung tissue under low-grade, chronic inflammatory stress.

Then, the effect of PMA on NHLFs was tested. In particular, immunostaining was performed using cytokeratin 7, vimentin, and counterstained with actin and DAPI. Cytokeratin 7, marking epithelial cells (A549), and vimentin, marking fibroblasts, allowed for the clear identification and colocalization of both cell types within the matrix (4.20, 4.21). Throughout the culture period, no significant overexpression was observed in either marker compared to controls, suggesting a stable cell population and a balanced co-culture.

Upon treatment with PMA, fibroblasts exhibited notable morphological changes, shifting to a more stellate structure after 7 days, indicative of potential activation. This activation is further supported by increased vimentin expression observed at 14 days, with fibroblasts appearing elongated and forming prominent, thick fibers within the matrix. Such a response in morphology and vimentin expression could imply fibroblast activation, where cells transition towards a myofibroblastic phenotype associated with wound healing or fibrotic processes, marked by cytoskeletal reorganization and increased contractility [38].

This change suggests that PMA, through its stimulation of pro-inflammatory pathways, may simulate conditions that lead fibroblasts to adopt a remodeling role within the 3D matrix. This feature, in turn, highlights the capacity of the 3D construct to provide a dynamic and interactive environment where cellular responses to inflammatory stimuli can be closely observed and analyzed in the context of tissue repair and fibrosis.

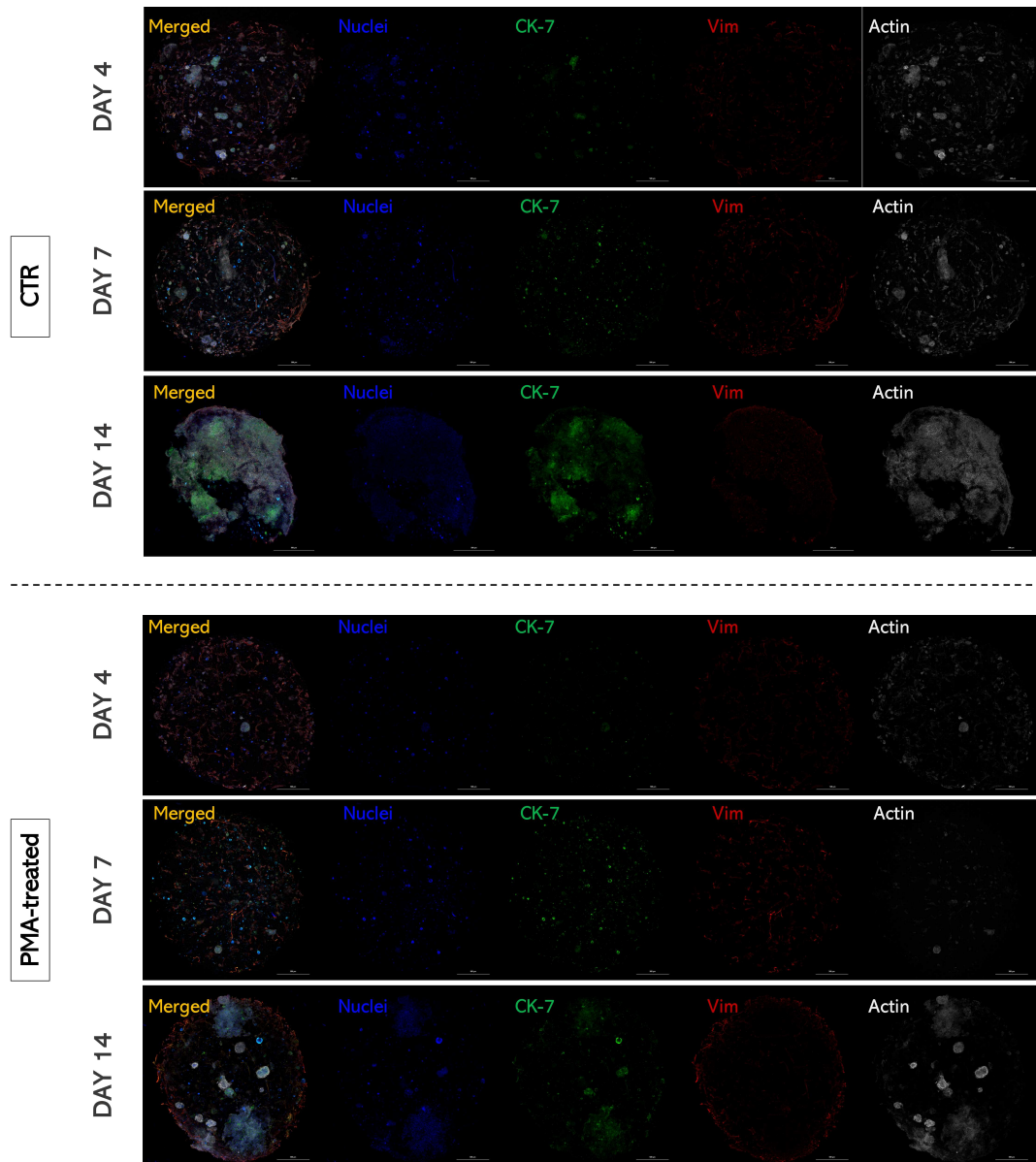


Figure 4.20: Representative immunostaining images of 4X confocal microscopy acquisition on 3D alveolar tissue constructs after 4, 7 and 14 days from PMA treatment (untreated samples acting as CTR). In red, the Vimentin over-expressed by activated NHLFs, in green the CK-7 to mark epithelial cells; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: 500 μ m.

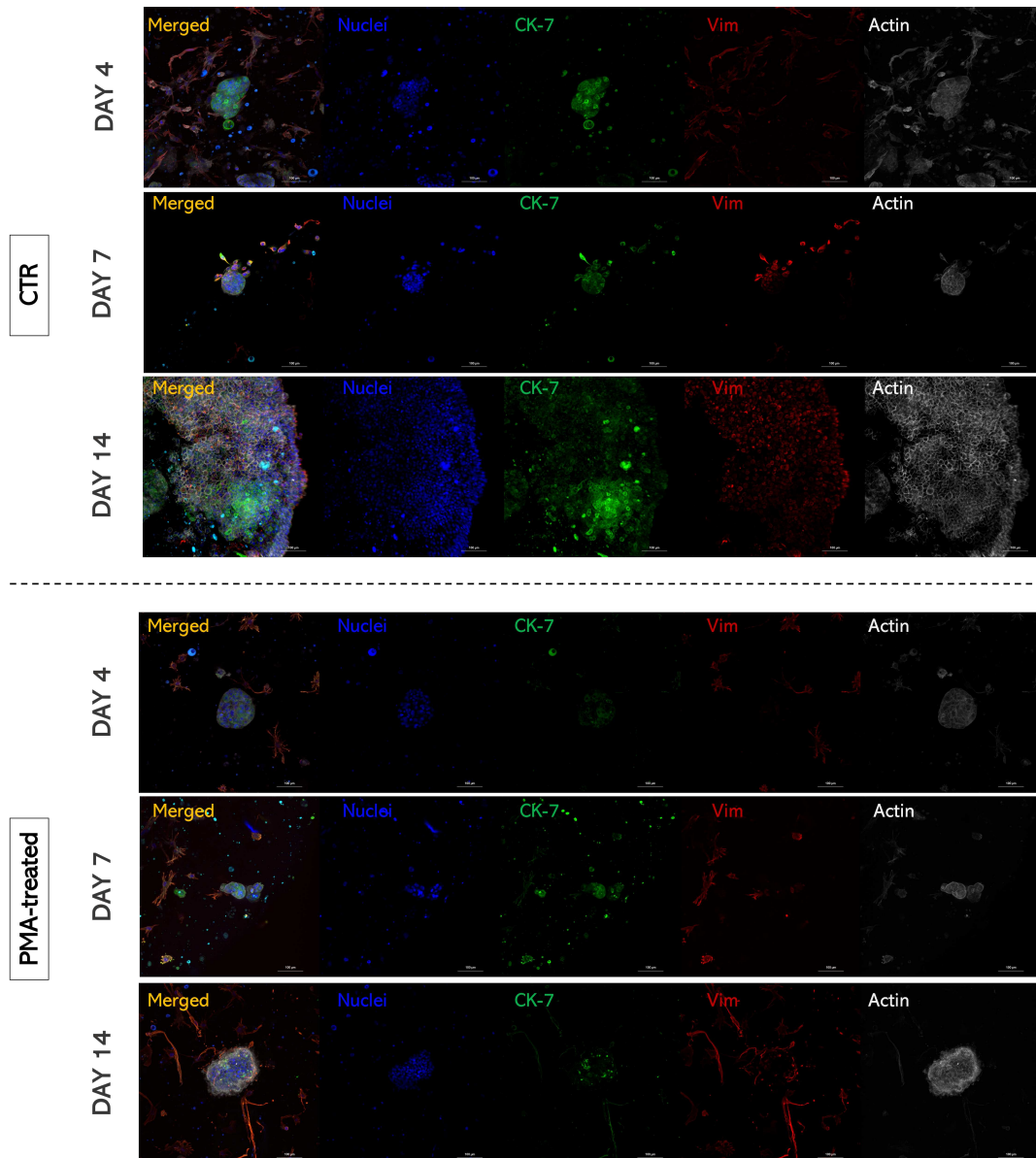


Figure 4.21: Representative immunostaining images of 10X confocal microscopy acquisition on 3D alveolar tissue constructs after 4, 7 and 14 days from PMA treatment (untreated samples acting as CTR). In red, the Vimentin over-expressed by activated NHLFs, in green the CK-7 to mark epithelial cells; cells were counterstained with phalloidin (actin filaments, in white) and DAPI (cell nuclei, in blue). Scale bars: 100 μ m.

Results were further analyzed by histology. In particular, Alcian Blue and Picrosirius Red staining provided insight into ECM deposition and remodeling, particularly after PMA treatment. Alcian Blue staining revealed an intensified blue coloration after 7 and 14 days, with the treated samples showing even greater staining intensity, suggesting increased glycosaminoglycan (GAG) deposition, likely of sulfated GAGs such as chondroitin sulfate and HA.

These GAGs are often upregulated in inflammatory conditions and contribute to ECM hydration and structure, indicating that the PMA treatment may be enhancing matrix remodeling processes typically associated with alveolar repair responses [39].

Picrosirius Red staining demonstrated an increase in red-stained collagen fibers, particularly after 14 days in PMA-treated samples. This staining reflects collagen fibrillogenesis, especially involving collagen types I and III, which are commonly associated with tissue stiffness and fibrosis under inflammatory conditions. The architecture of the 3D scaffold, however, constrained the orientation of collagen fibrils, limiting directional fiber analysis [40]. Despite this limitation, the scaffold's porous structure, which directs collagen deposition, suggests that the observed collagen build-up likely plays a structural role within the construct, as it could contribute to matrix organization and cell-matrix interactions over time.

These results suggest that PMA treatment might induce fibroblast activation, leading to ECM deposition patterns that mirror those seen in fibrotic or reparative lung tissue, where GAGs and collagen are overexpressed. This histological profile reinforces the suitability of this model for studying ECM remodeling and cellular responses in a controlled alveolar-like environment, providing a valuable system to assess experimental lung therapies for fibrosis.

Now a macroscopic effect of remodelling after NHLFs activation in terms of mechanical properties was investigated through rheology. Rheological analysis after PMA treatment, demonstrated notable changes in the mechanical properties that align with typical responses seen in fibrotic or inflamed tissue. Strain sweep tests indicated a reduction in the critical strain (γ_C almost halved from 5.2% to almost 2.7%) defining the linear viscoelastic region (LVR), which may reflect a shift towards a less elastic, more brittle ECM characteristic of fibrotic tissue (**Figure 4.23 A**). This shift suggests that PMA treatment might be promoting matrix densification and stiffening, potentially linked to collagen and GAG deposition.

Furthermore, both storage (G') and loss (G'') moduli showed higher values in PMA-treated samples at day 7, indicating increased rigidity and viscous dissipation in the construct. These heightened moduli suggest the formation of a denser, stiffer network in response to PMA-induced fibroblast activation (**Figure 4.23 B**), where the increased presence of ECM components like collagen could contribute to enhanced rigidity.

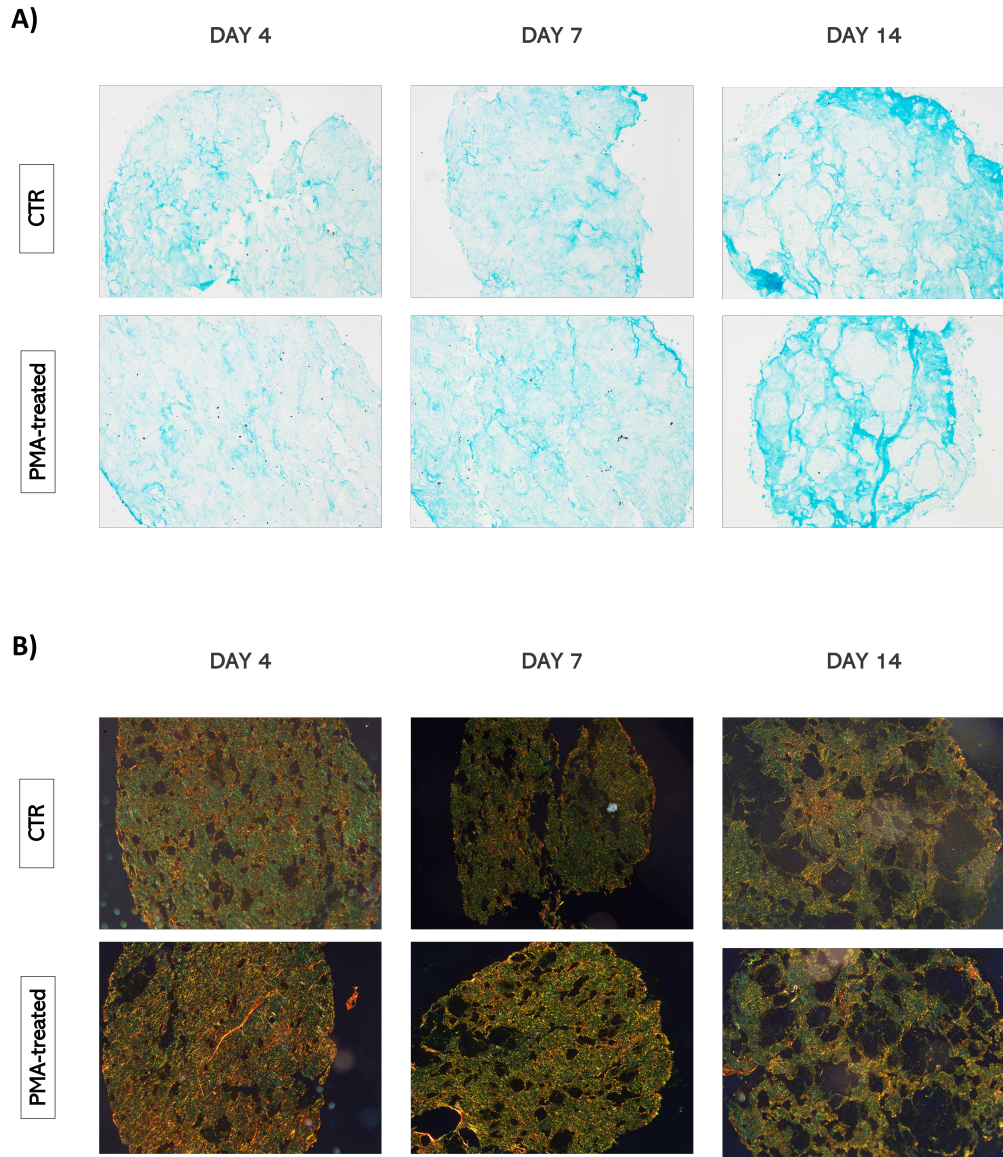


Figure 4.22: Representative histological sections acquisition by microscopy on 3D alveolar tissue constructs after 4, 7 and 14 days from PMA treatment (untreated samples acting as CTR). **(A)** Alcian Blue staining allowed the identification of GAGs deposition (in dark cyan) before and after the inflammatory insult. **(B)** Picosirius red polarization method was used as selective histochemical procedure for collagen detection. The green/yellow-ish sections of each image have to be interpreted as the less packed Coll-III fibrils, while Coll-I, characterized by thicker and more organized fibrils, appears in bright yellow, orange, or red hues and represents the collagen portion remodeled by stromal cells.

Interestingly, $\tan(\delta)$ (i.e. G''/G') values were slightly higher in PMA-treated samples, though not significantly, pointing to a minor increase in the viscoelastic damping response of the construct.

Collectively, these rheological changes suggest that PMA treatment may be inducing a fibrotic-like response, with ECM remodeling and stiffening that align with the mechanical behavior seen in inflamed or fibrotic lung tissue. Such adaptations underscore the model's value for studying tissue stiffening and the mechanical impact of therapeutic agents within a 3D alveolar matrix, offering insights into ECM dynamics under pathological conditions.

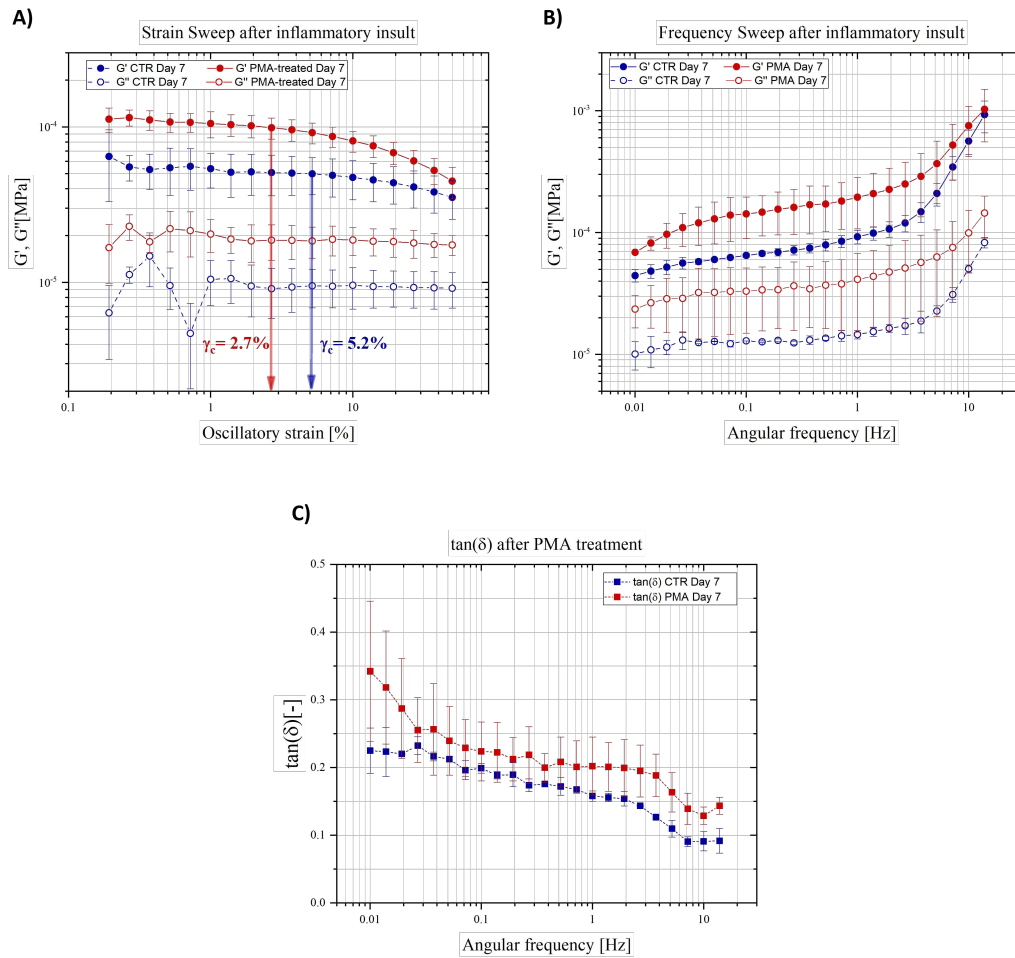


Figure 4.23: Rheological characterization of 3D alveolar tissue constructs pucks 7 days from PMA treatment (untreated samples acting as CTR). **(A)** Strain sweeps after the fibroblasts activation following PMA treatment. G' modulus in the plot show a reduced critical strain deformation (γ_c reported as arrows on the graph) for fibrotic constructs. **(B)** Frequency sweeps following PMA treatment show a higher value for both moduli G' and G'' in the whole frequency range for fibrotic constructs. **(C)** Effect of PMA treatment on $\tan(\delta)$ during frequency sweep characterization.

4.3.9 Conclusions and future perspectives

In conclusion, the development of a 3D biomimetic alveolar system was crucial for overcoming the limitations posed by traditional 2D and non-biomimetic *in vitro* models, which often fail to replicate the complex cellular interactions and microenvironment of distal lung tissue. The need for such a model stems from the high failure rates observed in translating experimental therapies from *in vitro* models to clinical applications, where the simplified architecture and lack of spatial cell localization are often inadequate to predict therapeutic efficacy and safety.

In this work, a novel 3D alveolar model was designed to incorporate both NHLFs and A549 epithelial cells within a Col-HA biomimetic hydrogel matrix. The synthesis process involved emulsification of droplets encapsulating A549 cells, which were successfully integrated into the ECM-like matrix, retaining cell viability over extended culture periods.

This biomimetic encapsulation in the Col/HA matrix allowed for the creation of a stable 3D environment that, upon hydrogel dissolution, provided a complex network for cellular interactions and spatial organization of both cell types. Unlike previous models, which have often lacked clear colocalization between fibroblasts and epithelial cells, this system successfully established a multicellular alveolar construct with defined cellular positioning.

Moreover, this 3D alveolar model demonstrates potential as a versatile platform for studying the responses of distal lung tissue in both healthy and inflamed states, as shown by treatment with PMA to mimic inflammatory conditions. The model's responsiveness to inflammatory stimuli highlights its suitability for studying pathophysiological processes such as ECM remodeling and cell activation in a more realistic, *in vivo*-like environment. This feature allows the system to serve not only as a research tool for studying lung pathologies but also as a test bed for experimental therapies targeting inflammatory pathways, cellular cross-talk, and tissue repair in alveolar environments.

Another essential property resides in the scalability. The procedure allows the 3D alveolar structure to be easily scaled up, in order to produce bulky samples, used for example in mechanical testing, still replicating the same architecture and cells disposition. Then, the porous structure still allows a high viability of the constructs in the inner core, since nutrients and oxygen diffusion are not hindered by a thick membrane diffusion path.

Looking forward, this system holds promising avenues for further development. One possible future perspective could be the introduction in the system of other cell types, eventually integrating alveolar macrophages, allowing for a closer analysis of inflammatory reactions and immune signaling within the lung tissue model.

Additionally, introducing endothelial cells could offer insight into vascular interactions, enhancing the physiological relevance of the model for some disease studies, lacking at this moment when having to model pathologies in which immune and endothelial cells critical roles in tissue repair, such as ARSD, COPD or asthma.

Furthermore, it can be further refined in terms of synthesis to support the encapsulation of more delicate primary epithelial cells, offering a closer approximation to human lung tissue. Moreover, by employing patient-derived cells, this model could be adapted for personalized medicine, allowing researchers to study patient-specific responses to new therapies and tailor treatments based on individual inflammatory and cellular profiles. With optimization, this biomimetic alveolar model has the potential to revolutionize lung tissue engineering, advancing both regenerative medicine and disease modeling.

Conclusions and future perspectives

In conclusion, this PhD thesis work demonstrated the versatility of natural materials in lung tissue engineering applications, highlighting their adaptability to diverse regenerative and engineering needs.

As a matter of fact, the first part of this thesis focused on developing a microstructured platform of natural polymers loaded with MSCs in a biomimetic, biphasic suspension designed to promote lung tissue repair. By using collagen microspheres without chemical crosslinkers, MSCs could be delivered sustainably and encouraged to differentiate into ATII cells, enhancing targeted retention and adherence at damaged lung sites. This platform, suspended in a LMWHA solution, holds promise for reversing lung tissue damage through optimized MSC release and site-specific adhesion.

Building on this regenerative platform, the thesis then addressed the impact of lung infections that contribute to chronic lung damage. FA-based CDs were developed as a biocompatible, antimicrobial nanoparticle system, leveraging their tailored surface chemistry to address drug-resistant bacteria that exacerbate lung deterioration.

The CDs' antimicrobial activity, particularly against bacterial strains relevant to lung infections, was directly correlated with their synthesis-dependent structural properties, demonstrating their potential in fighting infection-associated inflammatory damage.

Finally, the synthesis of a 3D biomimetic alveolar construct using a collagen and hyaluronic

acid hydrogel was developed, incorporating A549 and fibroblast cells for the first time in an encapsulated environment that ensures long-term cellular viability and spatial localization. This construct, capable of simulating both healthy and inflamed distal lung tissue, provides an innovative platform for studying inflammatory pathways and responses to experimental treatments.

Altogether, the results validate the adaptability of natural compounds, from supporting lung regenerative medicine with MSC delivery to combating infections and fabricating complex, biomimetic constructs for alveolar engineering, with future potential for use in personalized medicine applications. Looking ahead, several exciting avenues for future research can be envisioned. One promising direction involves integrating patient-derived cells into this 3D construct holds significant potential for personalized medicine, allowing for tailored therapeutic approaches that align with individual patient profiles and responses.

Additionally, the application of the developed carbon dots in conjunction with the 3D construct opens up innovative possibilities for treating lung infections. Testing the antimicrobial efficacy of the CDs within the biomimetic alveolar model could provide valuable insights into their effectiveness against specific bacterial strains and their potential to modulate inflammatory responses.

In addition FA-CDs could be interestingly used to study their bioimaging capability by a coculture system with healthy and cancerous epithelial cell lines. Such system would allow to model one of the main problem faced in current bioimaging: the lack of a 3D ECM-like structures hindering FA-CDs uptake by foalte receptors-expressig tumor cells, as in *in vivo* studies.

This combined approach not only enhances the construct's functionality but also addresses the pressing issue of antibiotic resistance. By exploring these perspectives, future studies can further advance the field of lung tissue engineering, paving the way for more effective therapies and strategies to combat lung diseases.

Nomenclature

Acronyms (Alphabetical order)

<i>(h)UCMSCs</i>	(human) Umbilical Cord Mesenchymal Stem Cells	<i>GAGs</i>	Glycosaminoglycans
<i>AB</i>	Alamar Blue (assay)	<i>Gly</i>	Glycine
<i>ARSD</i>	Acute respiratory distress syndrome	<i>HA</i>	Hyaluronic Acid
<i>ATI/ATII</i>	Alveolar Cell type I/II	<i>iBAQ</i>	Intensity-based absolute quantification
<i>BPD</i>	Bronchopulmonary dysplasia	<i>IPF</i>	Idiopathic pulmonary fibrosis
<i>BSA</i>	Bovine Serum Albumin	<i>LTE</i>	Lung Tissue Engineering
<i>CDs</i>	Carbon dots	<i>MDR</i>	Multidrug resistance
<i>CLSM</i>	Confocal Laser Scanning Microscopy	<i>MSCs</i>	Mesenchymal Stem Cells
<i>Col-x</i>	Collagen type x	<i>OMS</i>	Organizzazione Mondiale della Sanità
<i>COPD</i>	Chronic obstructive pulmonary disease	<i>PAH</i>	Pulmonary Arterial Hypertension
<i>DAPI</i>	4',6-diamidino-2-phenylindole	<i>PBS</i>	Phosphate-Buffered saline
<i>DMEM</i>	Dulbecco's Modified Eagle Medium	<i>PGs</i>	Proteoglycans
<i>ECM</i>	ExtraCellular Matrix	<i>Pro</i>	Proline
<i>EDTA</i>	Ethylenediaminetetraacetic acid	<i>SAGM</i>	Small Airway Epithelial Cell Growth Medium
<i>EMEM</i>	Eagle's Minimum Essential Medium	<i>SPC</i>	Surfactant protein C
<i>FBS</i>	Fetal Bovine Serum	<i>STE</i>	Soft Tissue Engineering
		<i>WHO</i>	World Health Organization

Alg	Alginate	-CD	-Cyclodextrin
ALIs	Acute lung injuries		
ATMSCs	Adipose tissue derived mesenchymal stem cells	<u>Samples and materials (Alphabetical order)</u>	
BMMSCs	Bone marrow derived mesenchymal stem cells	AA	L-ascorbic acid
EDTA	Ethylenediaminetetraacetic acid	AB	Alamar Blue
ESCs	Embryonic stem cells	ATR-IR	Attenuated total reflection infrared (spectroscopy)
FA	Folic acid	BSA	Bovine serum albumin
FGFs	Fibroblast growth factors	CK-7	Cytokeratin-7
HBECs	Human bronchial epithelial cells	Col/HA	3D methacrylated collagen and thiolated hyaluronic acid gel
Hyp	Hydroxyproline	DAPI	6-diamidino-2-phenylindole
iPSCs	Induced pluripotent stem cells	DTG	Differential thermogravimetric analysis
LOC	Lung-on-chip	EMEM	Eagle's Minimum Essential Medium
LVR	Linear viscoelastic region	FA-CDs	Folic acid-based carbon dots
MBMSCs	Menstrual blood derived mesenchymal stem cells	FBS	Fetal Bovine Serum
MW	Molecular weight	FT-IR	Fourier transform infrared (spectroscopy)
NHLFs	Normal human lung fibroblasts	HT/CDs	Carbon dots obtained after 10 mins microwave irradiation at 185°C
PFA	Paraformaldehyde	IL-x	Interleukin x
PMP	Poly(4-methyl-1-pentene)	LT/CDs	Carbon dots obtained after 10 mins microwave irradiation at 130°C
ROS	Reactive oxygen species		
TEER	Trans-epithelial electrical resistance		
WJMSCs	Wharton's Jelly derived mesenchymal stem cells		

mAlgs	Blank alginate microbeads	TGA	Thermogravimetric analysis
mAlgs/A549	A549-loaded alginate microbeads	TNF-	Tumor necrosis factor
		Vim	Vimentin
mCOLLs	Microspheres made of self-assembled type I collagen	ZO-1	Zonula Occludens-1
MMC	Mitomycin C	-MEM	Minimum essential medium with the alpha modification
MT/CDs	Carbon dots obtained after 10 mins microwave irradiation at 170°C	-MEM/HA	-MEM with 5 mg/ml LMWHA
MWCO	Molecular weight cutoff		
PA	Pseudomonas aeruginosa	<u>Variables (Alphabetical order)</u>	
PBS	Phosphate buffer saline	$\dot{\gamma}$	Shear rate
PBS + mCOLLs	PBS with mCOLLs in suspension	η	Viscosity
PBS + MSCs	PBS with 1×10^6 MSCs/ml	η_0	(Ellis and Cross fluid model) zero-shear viscosity
PBS/HA	PBS with 5 mg/ml LMWHA	η_∞	(Cross fluid model) Terminal viscosity
PBS/HA + mCOLLs	PBS with 5 mg/ml LMWHA and mCOLLs in suspension	η_C	(Alginate rheology) Viscosity of continuous phase
PBS/HA + MSCs	PBS with 5 mg/ml LMWHA and 1×10^6 MSCs/ml	η_D	(Alginate rheology) Viscosity of dispersed phase
PMA	Phorbol 12-myristate 13-acetate	η_s	solvent viscosity
r-mAlgs	Fluorescently red-labelled alginate microbeads	η_{sp}	Specific viscosity
		γ	Shear strain
RO	Rapeseed oil	γ_C	Critical shear strain
SA	Staphylococcus aureus	λ	(Ellis-fluid model) relaxation time of the polymer solution
SAGM	Small Airway Epithelial Cell Growth Medium	σ	Standard deviation

σ_0	Standard deviation at day 0	n (Empirical model)	number of data
τ	Shear stress	O_1	molar extinction coefficient of oxidized AB at 570nm
ξ	Gel's mesh-size	O_2	molar extinction coefficient of oxidized AB at 600nm
ζ	Surface zeta potential	p (Empirical model)	degrees of freedom
A_1	absorbance of test wells at 570 nm	P_1	absorbance of positive growth control well at 570nm
A_2	absorbance of test wells at 600 nm	P_2	absorbance of positive growth control well at 600 m
C^*	overlap concentration	$\tan\delta$	Ratio of the two portions of the viscoelastic behavior
C^e	entanglement concentration	ANOVA	analysis of variance
D (Empirical model)	Measured mCOLLs diameter	IQR	Interquartile range
D (Meshsize equation)	Solute diffusion coefficient in the hydrogel	k (empirical model)	rate of exponential decay
D_0 (Dimensional distribution)	Measured mCOLLs diameter at day 0	k (Cross-fluid model)	relaxation time of the polymer solution
D_0 (Meshsize equation)	Solute diffusion coefficient in water	p (Alginate rheology)	Viscosity ratio
D_∞ (Empirical model)	stabilized diameter	q_1	First quartile (statistical analysis)
d_i (Empirical model)	standardized residuals	q_2	Second quartile (statistical analysis)
G'	Shear storage modulus	SD (Statistical analysis)	Standard deviation
G''	Shear loss modulus		
n (Ellis and Cross fluid model)	flux index		

Appendix A

Critical gel theory

Below follows a brief discussion -without any pretense of completeness- on the theory of the critical gel, having as main reference works those of Winter and Chambon [1, 2]. Their works have been used as a guide line in the analysis of the rheological properties in **chapter 4**.

In fact, systems exist which face a transition from a viscoelastic liquid-like behavior to a viscoelastic solid-like one, as a function of variables such as temperature, pH or polymerization time. When passing from liquid to solid they go through a state called *critical gel state*. Depending on the variables involved and the reversibility of the transition, it's possible to identify of physical gels or chemical gels:

- A *chemical gel* is not reversible and is made up of chemical bonds, called *crosslinks*), that give rise to a three-dimensional network which do not allow to return to the initial state, once the initial conditions have been re-established. Long-range connectivity is given by covalent bonds between molecules and it is possible to identify a single one molecular cluster with dimensions diverging to infinite;
- A *physical gel* is reversible and is constituted by physical bonds, such as hydrogen bonds or other inter-molecular forces that result in a long-range form of self assembly. These gels are, in fact, capable of giving rise to supra-molecular aggregates through the large number of mechanisms.

In both cases, the system is made up of molecules not bound to each other, but free to move (liquid-like behavior). Following the variation of one of the parameters listed above, this system leads to a state in which the molecular mobility is strongly reduced (solid-like behavior). As a matter of fact, in the case of the liquid, chains are entangled, but nevertheless free to flow with respect to each other and therefore they can flow over long time scales.

After that, the gelling process consists in starting to bind the molecules to each other: therefore connectivity increases, up to the formation of a three-dimensional network that affects the whole system. This condition is called, in fact, of critical gel, because it is a very labile condition. In fact, if a link is broken, the system is able to flow again, returning to a liquid-like behavior.

On the contrary, with the formation of a further bond, the system manifests a stable structure that gives solid-like behavior. It is possible to follow the trend of this phase transition by studying the variation of measurable parameters (viscosity and modulus of equilibrium) as a function of the degree of conversion, the trend of which is described in **A.1**.

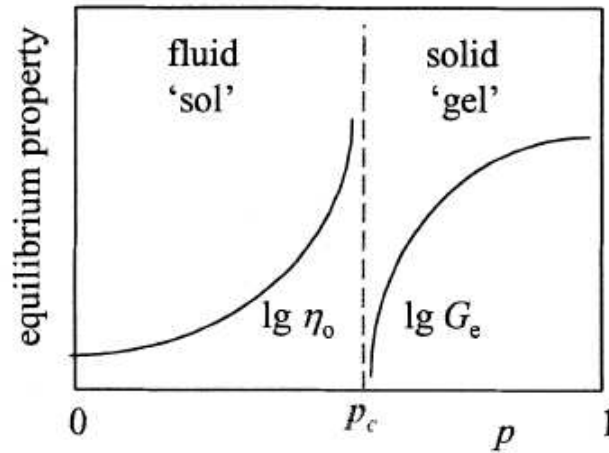


Figure A.1: Schematic plot of the viscosity of *zero shear* η_0 and of the equilibrium module G_e as the degree of conversion p varies.

At first, the system is liquid, so it will have a viscosity of *zero shear* (η_0). However, when bonds begin to form between molecules, the viscosity increases, until it diverges when the solid state is reached, in which viscosity is by definition infinite.

If, on the other hand, we evaluate the *equilibrium module* (G_e), in the case of the liquid it will be zero, since it is not possible to speak of an elastic equilibrium in a material capable of flowing; while from the moment in which the solid is formed, the system no longer flows, and therefore we begin to notice an elastic equilibrium modulus.

In particular, the two rheological functions used are defined by:

$$\eta_0 = \lim_{\substack{t \rightarrow \infty \\ \dot{\gamma} \rightarrow 0}} \eta(\dot{\gamma}, t) \quad (\text{A.1})$$

$$G_e = \lim_{t \rightarrow +\infty} G(t) \quad (\text{A.2})$$

The point that marks the transition from liquid to solid, the one in which these two quantities lose all physical significance, is precisely the critical gel point. However, we want to plot the viscoelastic properties of the material as a function of variables, with respect to which these properties do not diverge near the gel point ¹.

Another identifying characteristic of the gel point is the *self-similarity* (or *scale invariance*) [3] property, i.e. the ability to relax according to a law of type *power law* along a more or less large time window. The relaxation spectrum $H(\lambda)$ of such a polymer, i.e. the function that defines its linear viscoelastic behavior, will be given by **Equation A.3**.

$$H(\lambda) = \frac{S_c}{\Gamma(n_c)} \lambda^{-n_c} \quad \text{per } \lambda > \lambda_0 \quad (\text{A.3})$$

Where the following quantities appear:

- The *Euler gamma function*, which has the property of recursion and which can be approximated to a parabola:

$$\Gamma(n_c) = \frac{1}{n_c} \Gamma(1 + n_c) \approx \frac{1}{n_c} [1 - 0.01138(1 - 4(n_c - 0.5)^2)] \quad (\text{A.4})$$

¹This idea would seem to find fertile ground in the observation of the fact that the rheology during the gelation process it has the same characteristics for physical and chemical gelation.

- The *stiffness of the critical gel* - whose dimensions are homogeneous to a product between the characteristic module G_0 and the characteristic time λ_0 - given by:

$$S_c = \frac{G'(\omega)}{\omega^{n_c} \Gamma(1 - n_c) \cos \frac{n_c \pi}{2}} \quad (\text{A.5})$$

- The *relaxation exponent*, a frequency independent constant:

$$n_c = \frac{2\delta}{\pi} = \frac{2 \arctan(\frac{G''}{G'})}{\pi} \quad (\text{A.6})$$

This property of the relaxation spectrum of a critical gel can be well observed starting from a bilogarithmic plot of the relaxation modulus $G(t)$ as a function of time (**Figure A.2**).

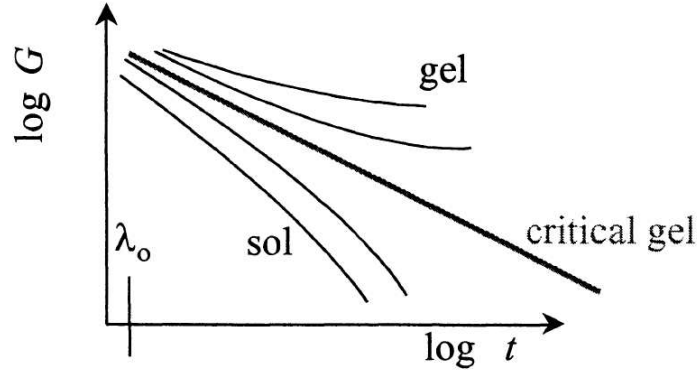


Figure A.2: Relaxation modulus over time as the condition of the polymer varies from the *sol* state, to that of a critical gel, to that of *gel*. We can observe for $\lambda > \lambda_0$ the self-similarity of the relaxation spectrum.

Among all quantities, the ones defined in **Equation A** and in **Equation A.5**, are able to give information -once extrapolated from experimental data- on the type of gel with which you are dealing, in particular if you are dealing with a *soft gel* or with an *hard gel* (**Figure A.3**).

Among the theories of physical chemistry that focus on crosslinking, the *Flory-Stockmayer theory* is among the first to describe the evolution of the system structure; other structural information can be obtained from percolative theories or mean field theories.

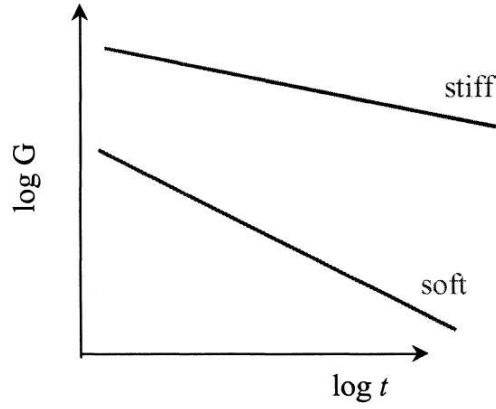


Figure A.3: Comparison between a *soft gel* (with $n_c \rightarrow 1$ and S_c small) and a hard gel (or *stiff gel* with $n_c \rightarrow 0$ and S_c large)

As previously noted, it is possible to monitor the progress of the crosslinking phenomenon by the degree of chemical conversion ($0 \leq p < 1$). In particular, having defined the p_c threshold value as previously reported, below the critical gel point ($p < p_c$), the crosslinking sample is called *sol*, as it is capable of solubilize in a good solvent.

On the other hand, above the critical gel point ($p > p_c$) the polymer can swell only in a good solvent, even if it does not dissolve. The state the polymer is in is therefore called *gel*. In this terminology, the transition the polymer undergoes from liquid-like to solid-like is often called *sol-gel transition*.

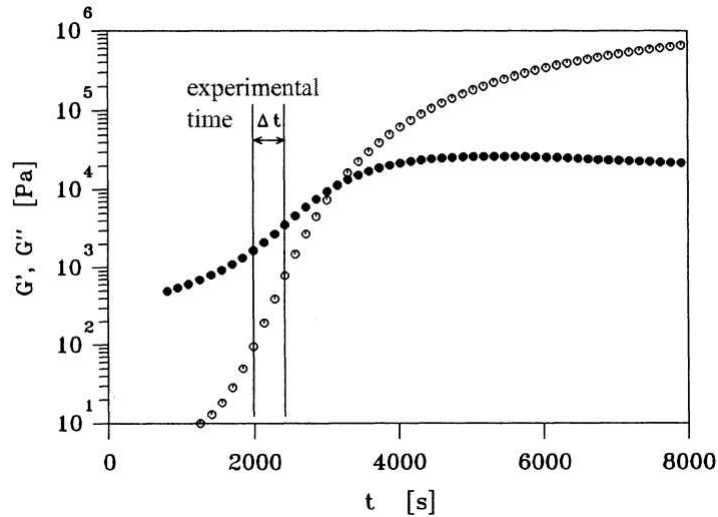


Figure A.4: Typical frequency response in terms of modules $G'(\omega)$ and $G''(\omega)$: from a plot of this type we can go back to mutation time λ_{mu} .

A typical rheological response in terms of *loss modulus* G'' and *storage modulus* G' over time is reported in **Figure A.4**.

Clearly, in the first moments of the test it's possible to observe a loss modulus higher than the storage modulus, indicating a liquid-like behavior; this trend is reversed once a crossover point is passed.

What has to be taken into consideration is that the measured mechanical properties are extremely sensitive to structural changes the polymer undergoes over time, since new crosslink have been formed from one experimental point to another. As a matter of fact, it is possible to define a characteristic time of change in the material λ_{mu} (**Equation A.7**), i.e. the time required for a given module to increase by a e factor.

$$\lambda_{mu} = \left(\frac{1}{G'} \cdot \left| \frac{\partial G'}{\partial t} \right| \right)^{-1} \quad (\text{A.7})$$

The choice to use G' was motivated by the fact that the rate of growth of this magnitude is more appreciable than G'' . In addition to this characteristic time, information on the crossover time λ_0 is obtained from the plot above, a characteristic parameter of the specific polymer, which can be obtained - precisely - from the abscissa of the intersection of the curves of the two modules.

A.0.1 Analysis for a physical thermosensitive gel

What is done in the case of a physical gel of the thermosensitive type is to carry out *Temperature sweep* tests in which, once the oscillation and deformation frequency has been set, the trend of the two modules is storage modulus G' and the loss modulus G'' as the temperature changes.

A typical thermosensitive gel response can be observed in the samples: at low temperature values, $G'' > G'$ (index of liquid-like behavior), while rising with temperature will reach a point of crossover where the two modules coincide $G'' = G'$, which will be followed by an inver-

sion in the behavior of the material, as there will be $G' = G''$. Therefore, is possible to think that the temperature corresponding to the crossover of the two modules is the gel temperature T_{gel} .

As a matter of fact, it can be observed that, while maintaining the ramp speed unchanged, by conducting tests at different oscillation frequencies, this crossover point varies. This would mean that the gel temperature varies with the variation of the oscillation frequency at which the test is conducted, which is impossible, being a thermodynamic property of the material. Therefore, observing that in the vicinity of the critical gel point the G' and G'' modules continue in parallel with the variation of ω , it can be concluded that. $\frac{G''}{G'} = \tan\delta(\omega) = \text{const}$ (**Figure A.5**).

It is possible to represent $\tan\delta$ as a function of temperature, by parameterizing the representation at different frequencies, however below $\frac{1}{\lambda_0}$: what happens is that all the graphs converge in a point or at least in a determined range more or less narrow of temperatures. Ideally, the crossover point of such curves, where the value of $\tan\delta$ does not depend on frequency, gives the temperature of gel T_{gel} at the given ramp rate at which the test was conducted.

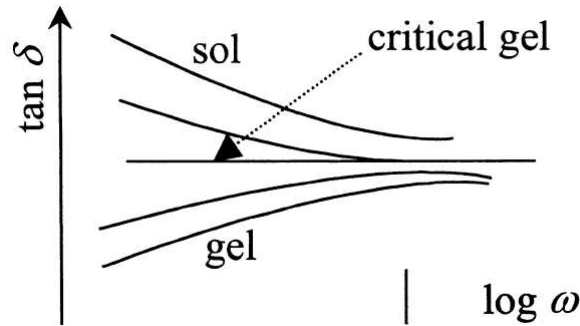


Figure A.5: Set of curves $\tan\delta(\omega)$ in the various instants of a gelation process: once the critical gel condition $\tan\delta(\omega)$ is reached, it assumes a flat course. It is also observed that for the same frequency ω , the value of $\tan\delta$ decreases as the gelation process progresses.

Finally, it remains to be discussed with regard to the kinetics of this gelation process: a more or less marked dependence of T_{gel} ², as well as of the typical hysteresis that is formed following the heating of the sample above the T_{gel} and its immediate cooling.

This latter phenomenon seems to fade when the test is carried out at speeds close to zero,

²The gel temperature, in fact, would seem to decrease significantly as the speed decreases of ramp $\frac{dT}{dt}$.

in a process that tends to thermodynamic equilibrium. In order to prove what has just been said, it's possible to plot the T_{gel} obtained at the different ramp speeds, as a function of the temperature variations, in order to be able to observe a trend with good linear approximation. Therefore, it is thus possible to extrapolate from the intercept on the ordinate axis the gel temperature that would be obtained if the tests were carried out in conditions of thermodynamic equilibrium, when $\frac{dT}{dt} = 0$.

A.0.2 Analysis for a chemically crosslinked gel

Tests to be carried out to characterize a chemical gel are extremely similar to those that can be used for a physical gel, bearing in mind that the variable that governs the process is no longer the temperature - at least not directly - but the time taken to form the crosslink. In fact, it is not possible to evaluate the gel time t_{gel} starting from the intersection of the two modules, but the quantity $\tan\delta$ must be considered as the time varies, always parameterizing the different curves at multiple frequencies $\omega < 1/\lambda_0$ (**Figure A.6**).

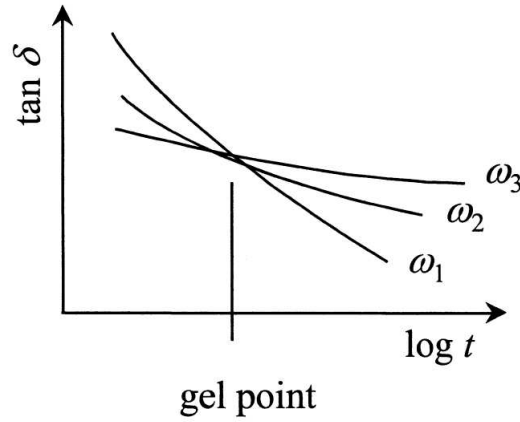


Figure A.6: Set of curves $\tan\delta(t)$ during a freezing process, parameterized in the frequency of the dynamic test. The intersection point of the curves marks the gel time t_{gel} .

A common problem to both physical and chemical type gels could be running into a certain polymer evidently having a particularly high crossover time, so that the range of frequencies to be investigated can be narrower somewhat.

A solution can be found, note that it is the constant n_c at least for that class of polymers. In fact, we can proceed to normalize the dynamic modules, thus evaluating the curve in **A.4** in reference, however, to the quantities $G'/\cos(n_c\pi/2)$ and $G''\sin(n_c\pi/2)$: in this case it would

therefore be legitimate to consider the abscissa of the crossover point as time t_{gel} (A.7).

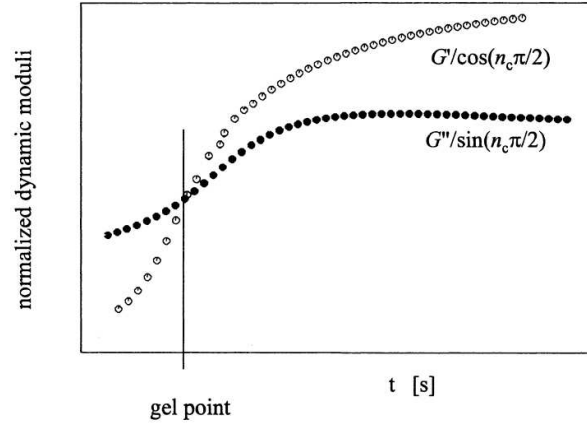


Figure A.7: Frequency response in terms of normalized modules $G'/\cos(n_c\pi/2)$ and $G''/\sin(n_c\pi/2)$: from a plot of this type it is possible to go back to t_{gel} , evaluating the crossover point.

It is now necessary to make a final reflection regarding the choice of monitoring a gelation of this type through the t_{gel} . In fact, since the crosslinking speed, as well as the gelation time, strongly depend on the temperature and on the heating and cooling kinetics of the sample, the time coordinate should be more correctly replaced with the degree of conversion p ³.

However, as far as this experimental work is concerned, it was not considered necessary to introduce such a complication, since the reference temperature for each test was set equal to the body temperature. Furthermore, there is no reason to imagine an application in which these sample can gel at temperatures very different from this one.

³Unlike the other parameters, the degree of connection within the polymeric network does not depend on the temperature and can be derived from the Flory-Stockmayer theory or from the Carothers equation.

Appendix B

Possible folic acid and urea degradation and reaction path to form FA-CDs

Here it is hypothesised a potential reaction path to the formation of FA-CDs from FA and urea, taking into account the results obtained by the precursors' TGA/DTG data reported in **subsection 3.3.4**. Starting from urea (**Figure B.1**), it is known that in aqueous solution at 152 °C urea gives hydrolysis, slowly equilibrating with Ammonium cyanate, in order to further convert to gaseous ammonia and isocyanic acid.

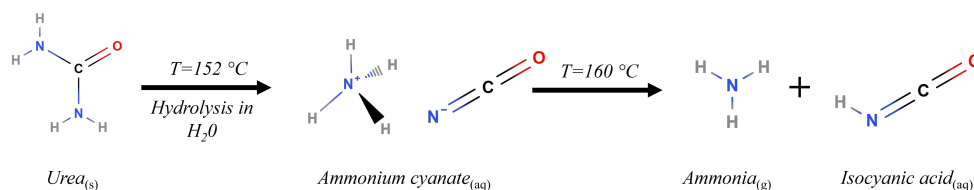


Figure B.1: Degradation path of urea molecule as a function of temperature.

On the other hand, on a study about FTIR analysis as a function of temperature, it was revealed that the first FA reaction is the loss of glutamic acid, followed by the loss of the amide, pterin. As a matter of fact, after absorbed water is lost at lower temperatures, the glutamic acid moiety is lost starting from 195°C.

from that point onward the other constituents of folic acid are degraded before they reach a temperature of 195°C. This means that after the complete loss of acid and amide functionality at 195°C, FA undergoes complete progressive degradation, resulting in an amorphous product above this temperature, from which CO_2 is freed and a graphitic trace is left.

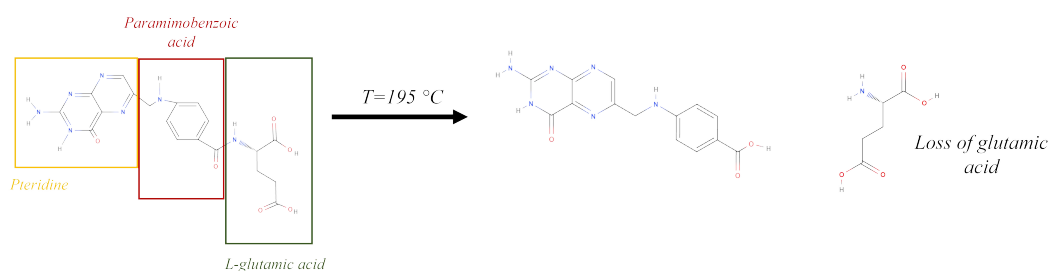


Figure B.2: FA molecular structure and degradation path as a function of temperature. Around 195 °C the loss of glutamic acid takes place and without any water molecule, the full degradation takes place for temperatures above 200°C.

Now, two cases should be considered: the one in which the reaction synthesis temperature is below the FA degradation temperature (corresponding to the LT/CDs and MT/CDs synthesis) and that one in which the temperature in the vessel reaches the degradation temperature (i.e. HT/CDs synthesis)¹.

This means that for LT/CDs and MT/CDs, the reaction precursor is the whole FA molecule (as in **Figure B.3** and **Figure B.4**), while the HT/CDs are obtained by a precursors solution in which the degraded FA -after the loss of glutammic acid moiety- reacts (as in **Figure B.5**). In all these situations an amide reaction can take place between the carboxyl and the amine groups, when temperature ranges between 110 and 140°C [2].

In particular, when the FA molecules are preserved, two possible reactions can take place, since they can react between themselves, giving polymerization or they can react with the urea dissolved in solution. In the first case, once two or three FA molecule interact via hydrogen bond, each of the two carboxylic groups on the L-glutammic acid moiety can react with the amino group on the pteridine residue of FA. For this reason, the product is a chain showing as terminal groups one amine and a total of four carboxyl moieties (**Figure B.3**).

¹It is worth to underline how the degradation temperature above-mentioned is evaluated in inert atmosphere, where the energy transfer occurs through conduction. In our case, instead, the presence of an aqueous solution and a microwave irradiation can have an effect on favoring FA degradation.[1]

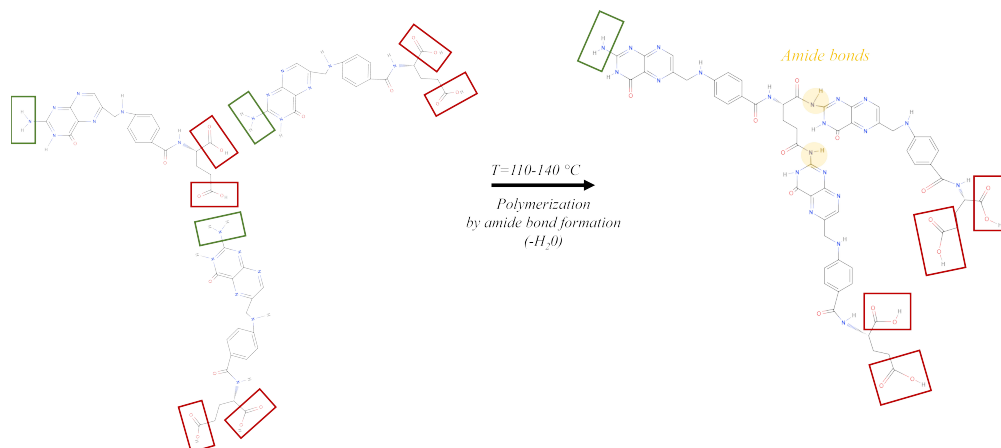


Figure B.3: Main reactions involving three molecules of FA, happening during polymerization below the degradation temperature. Amide bond formation happening between the two carboxyl groups at the end of the glutamic acid moiety of FA and one amine group from the urea. This reaction brings as product a molecule whose reactive sites are only amine groups.

However, in solution urea is present still as a whole molecule and therefore the condensation reaction can also happen with the two symmetric amide groups on urea². When this happens, another FA molecule can be connected, having urea as a "bridging molecule".

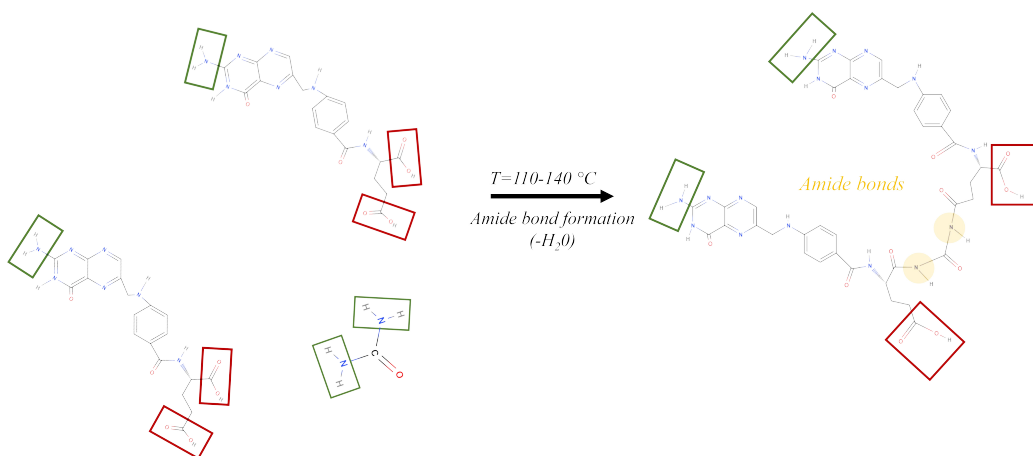


Figure B.4: Main reactions involving three molecules of FA, happening during polymerization below the degradation temperature. Amide bond formation happening between the two carboxyl groups at the end of the glutamic acid moiety of FA and one amine group from the urea. This reaction brings as product a molecule whose reactive sites are only amine groups.

Different from the previous set of reactions are those happening during the synthesis of HT/CDs, where the temperatures reached in the vessel correspond to those able to cause the loss of glutamic acid from the FA molecule. When this happens, the number of carboxyl moieties

²In particular, this reaction might be favoured from the point of view of steric hindrance, since urea molecule is far smaller, more flexible and with a higher diffusion coefficient compared to a whole FA molecule.

able to react with amine groups are cut by the half and the only polymerization reaction is the one occurring linearly across the molecule (**Figure B.5**).

As a matter of fact, the residue can still react through the carboxyl group belonging to the paramimobenzoic acid with another FA residue or a molecule of urea. However in both cases the reaction stops, because the two terminals are both amine groups. For this reason the only possible next reaction can be with another FA residue from the side of the carboxyl moiety. However it seem apparently a slower reaction route, compared to the other two, even though the hydrogen bonds interactions between the polymerized chains can still occur, bringing the molecule close enough to have a carbonization and water molecules release. That way, the typical graphene-like structure of CDs is again obtained.

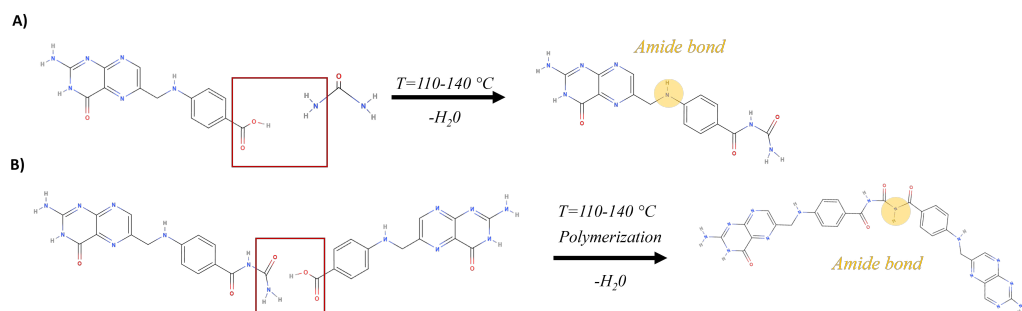


Figure B.5: Main reactions involving FA after the loss of glutamic acid and urea happening during polymerization around $195\text{ }^{\circ}\text{C}$. Amide bond formation happening between the carboxyl group of the paramimobenzoic acid part of FA and one amine group from the urea. This reaction brings as product a molecule whose reactive sites are only amine groups (**A**).

Appendix C

MATLAB code for 2d planar fit

```
1 % Load data from the file
2 arr = load('Zeta_pH_T.txt');
3 x = arr(:,1);
4 y = arr(:,2);
5 z = arr(:,3);
6
7 % Create the Design Matrix for linear regression
8 DM = [x, y, ones(size(z))]; % Design Matrix
9 B = DM\z; % Estimate Parameters using least squares
10
11 % Extract coefficients for the plane equation z(x,y) = a*x + b*y + c
12 a = B(1);
13 b = B(2);
14 c = B(3);
15
16 % Display the coefficients a, b, and c
17 disp(['Plane equation: z(x,y) = ', num2str(a), '*x + ', num2str(b), '*y + ', num2str(c)]);
18
19 % Generate grid points for plotting the plane
20 [X,Y] = meshgrid(linspace(min(x),max(x),100), linspace(min(y),max(y),100));
21 Z = a*X + b*Y + c; % Calculate Z values for the plane
22
23 % Plot the original data points in 3D
24 figure(1)
```

```

25 plot3(x, y, z, 'o', 'MarkerSize', 5)
26 hold on
27
28 % Plot the fitted plane
29 surf(X, Y, Z, 'FaceAlpha', 0.8, 'EdgeColor', 'none')
30 colorbar
31 xlim([min(x) max(x)])
32 ylim([min(y) max(y)])
33 zlim([min(z) max(z)])
34
35 % Equation of the line formed by the intersection of the plane with z=0
36 % Set z=0 in the plane equation: 0 = a*x + b*y + c
37 % Solve for y: y = (-a/b)*x - c/b
38 x_intersect = linspace(min(x), max(x), 100);
39 y_intersect = (-a/b)*x_intersect - c/b;
40
41 % Display the equation of the intersection line
42 disp(['Intersection line equation: y = ', num2str(-a/b), '*x + ', num2str(-c/b)]);
43
44 % Plot the intersection line on the 3D graph
45 z_intersect = zeros(size(x_intersect)); % Since it's the intersection with z=0
46 plot3(x_intersect, y_intersect, z_intersect, 'k-', 'LineWidth', 2)
47
48 % Finalize the 3D plot
49 grid on
50 xlabel('pH [-]');
51 ylabel('T [°C]');
52 zlabel('Zeta potential [mV]');
53 title('Zeta vs pH vs T with Fitted Plane and Intersection Line');
54 hold off

```

Listing C.1: MATLAB Code for used in Chapter 3 for the 2D Plane Fitting of the zeta potential data distribution as a function of pH and T

Appendix D

Thixotropic behavior of mAlgs suspension

In this section an interesting property of mAlgs suspension will be faced that can be further exploited for some future bioprinting applications, also in the context of a possible automatization for the 3D alveolar construct fabrication.

More in detail, as explained in **4.3.1**, after the fabrication and purification of mAlgs, the last step involved a quick centrifugation of the suspension through a $50\mu m$ cell strained at high rcf values. This led to the formation of a highly concentrated mAlgs suspension that was used to seed the mAlgs in the constructs.

Now, the handling of this jellylike suspension led to some qualitative observations that were subsequently inspirational in the investigation of the rheological properties of such a system. In particular, it was possible to describe the system as a:

- Highly concentrated microgel suspension: to emphasizes a suspension-like behavior with minimal free water, that allowed the flow of the system during pipetting through pipettes;
- Jammed microgel network: due to the dense, closely-packed structure that maintains a semi-solid, gelatinous character, in particular when is not put in flow, resembling a Bingham liquid with a *yield stress*.

For this reason, rheological investigations were carried out as explained in 4.2.7 in order to characterize the macroscopic behavior of such a system. In particular, oscillatory strain sweeps were performed on suspensions obtained from Alg solutions 2 and 3% w/v.

Preliminary oscillatory rheological tests showed some common characteristics of the mAlgs suspension. In particular, the rheological response in terms of strain sweep showed higher values for shear moduli in the case of 3% w/v (**Figure D.1A**). However, the critical strain for the LVR to NLVR transition shows no specific difference between the two suspensions. This is possibly due to the critical strain behavior determined mainly by the water *lamellae* in between the mAlgs and therefore independently of the polymer concentration in the mAlgs themselves.

Similarly in **Figure D.1B** it can be noticed a typical gel-like behavior from the two suspensions, with the G' and G'' moduli being parallel for both samples. However, the main difference with the 2% w/v sample is the frequency-independence of the two moduli, compared to the 3% w/v sample, where a non-zero slope can be highlighted. The main reason for such a deviation from the pure gel-like behavior (as also detected on 3D Alg gels in **Figure 4.6**) can be found in a slightly higher water retention capability of the 3% w/v sample, creating thicker water *lamellae* between the mAlgs.

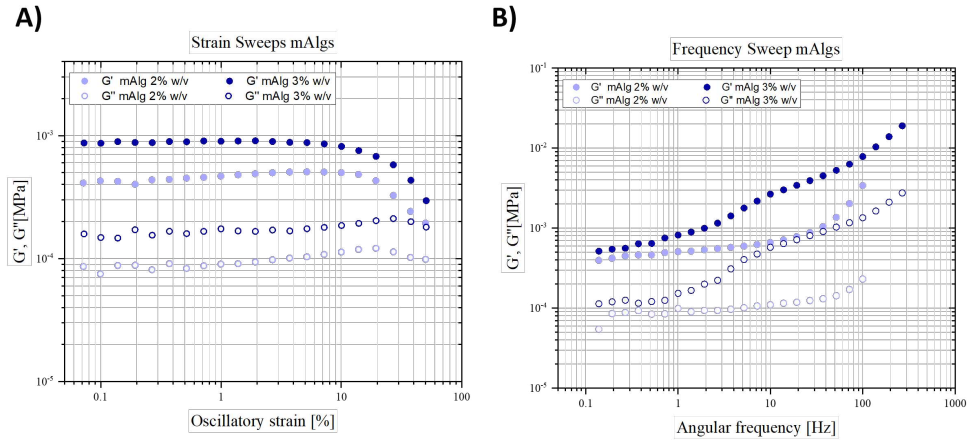


Figure D.1: Rheological investigation of the viscoelastic behavior under oscillatory conditions. **(A)** Strain sweep test on mAlg suspension derived from 2% and 3% w/v alginate solutions, showing the storage (G') and loss moduli (G'') as functions of strain. This test identifies the linear viscoelastic region and the critical strain point at which the structure begins to yield. **(B)** Frequency sweep test on mAlg suspension at 2% and 3% w/v alginate, displaying the storage and loss moduli across a range of frequencies.

A three-step oscillation technique was developed and utilized for analyzing thixotropy of the mAlgs suspension [1]. This oscillation technique is developed by utilizing three oscillation time sweeps at varying strain amplitudes, both within and beyond the sample's LVR.

Figure D.2 presents the results of oscillation measurements for the two samples. Initially, an oscillation time sweep is performed with a small strain of 0.1% that lies within the LVR. The sample exhibits a high complex modulus and the data indicates that the suspension possesses a structure, acting solid-like when at rest.

When the strain amplitude is raised to 100% during the second time sweep, the sample's structure is compromised. Samples moduli decline significantly, indicating that samples exhibit liquid-like behavior under elevated shear.

The third step time sweep is utilized to observe thixotropic recovery [2]. When the strain amplitude is reduced again to fall within the LVR, it requires a finite amount of time for the sample structure to restore. This recovery occurs in under 5 seconds for the 2% w/v sample and approximately 27 seconds for the 3% w/v sample after reaching LVR again.

The duration required for G' to achieve final equilibration represents the recovery time of the samples, and it is evident that both samples regain a significant portion of the moduli, with the 2% w/v sample almost exhibiting full recovery. This results preliminarily show that the 2% w/v sample exhibit a higher thixotropy with respect to the 3% w/v sample.

To further evaluate the thixotropic behavior of the mAlgs suspensions during the injection, a flow ramp up and down experiment was used. In this approach, also known as the thixotropic loop test, a shear stress or shear rate is increased from zero to a specific value at a certain pace, and then decreased back to zero at the same duration (**Figure D.3**).

In the rate ramp up phase, the sample structure is damaged, causing the downward curve to show hysteresis. The thixotropic index is determined by measuring the area between the ascending and descending curves [3]. Nonetheless, because this approach is a non-equilibrium ramp, the measurement outcome is significantly influenced by the rise and fall duration.

However, in this case the test parameters were kept constant for all the samples and results clearly showed a higher thixotropic degree for the 2%w/v mAlgs suspension ($8.43 \times$

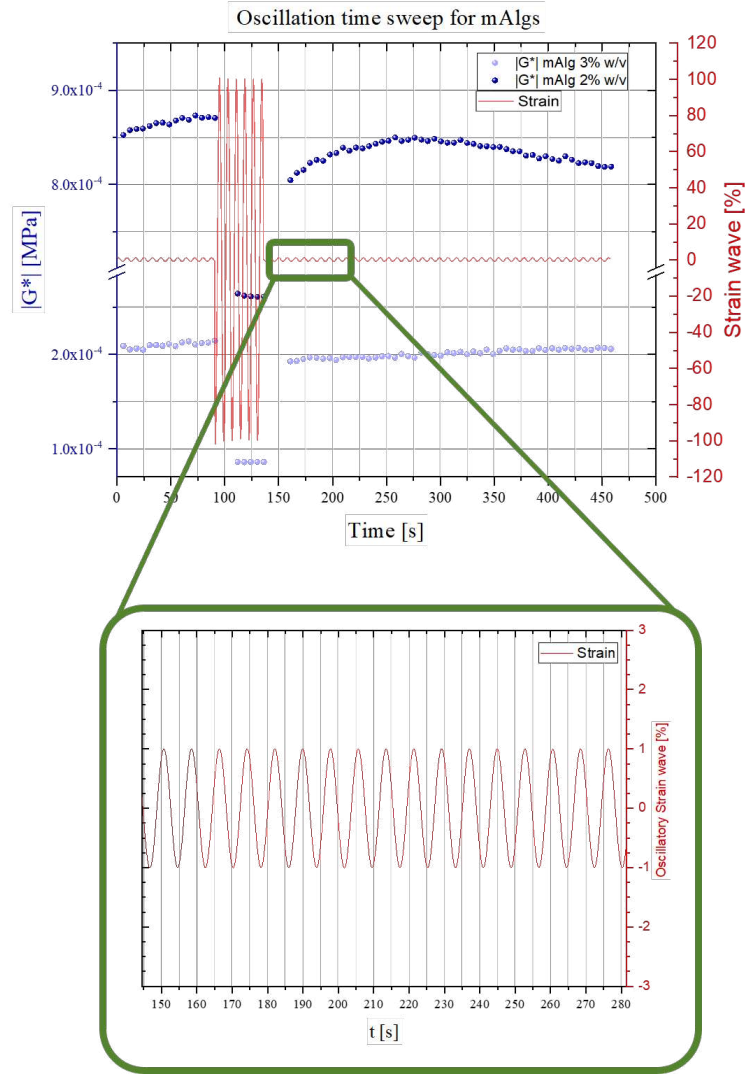


Figure D.2: Oscillatory rheology recovery test on mAlg suspensions derived from 2% and 3% w/v alginate solutions. The test protocol included an initial phase within the linear viscoelastic region (LVR) at 1% strain, followed by a structural breakdown phase at 100% strain, and a final recovery phase back at 1% strain, reported in the zoomed window. Results reveal the suspension's ability to rapidly restore its structure post-deformation, with the 2% formulation exhibiting near-complete structural recovery within approximately 5 seconds, while the 3% formulation achieved full recovery in around 27 seconds.

$10^{-3} Pa s$ versus the $4.49 \times 10^{-3} Pa s$ of 3%w/v sample), also in agreement with the modulus recovery data previously described.

Although this thixotropic loop test method does not give details about the recovery of the structure, it can yield a stable and clear response about samples' thixotropy that closely resembles what occurs during injection.

In the end, the flow behavior of the two different mAlgs suspensions can be determined via flow curves, investigating the dependence of viscosity on applied shear rate.

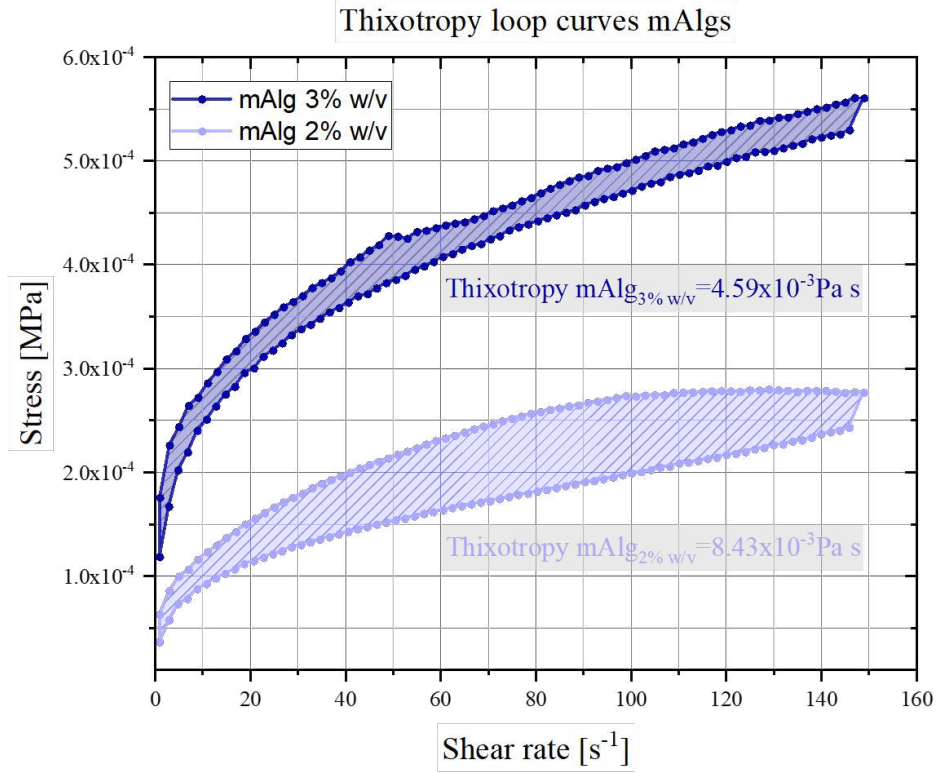


Figure D.3: Flow loop curve showing the thixotropic behavior of mAlg suspensions formulated from 2% and 3% w/v alginate solutions. The stress-shear rate curves demonstrate a hysteresis loop, with calculated thixotropic areas (area under the forward and return curves). These results illustrate the concentration-dependent thixotropy of the suspension, with the 2% formulation exhibiting a more pronounced hysteresis effect, indicative of greater structural rebuildability upon shear reduction.

It is known how as the concentration rises, the particles come closer, microgels suspensions become more densely packed and the fluid resistance against its moving layers increases resulting in high viscosities [4]. Moreover, a typical power law behavior can be noticed in the whole range of investigated shear rates.

In particular, power-law fits indicate their pseudoplastic nature, where apparent viscosity decreases with increasing shear rate. The 2%w/v sample shows a consistency index $k_{2\%w/v} = 141 \pm 2 \text{ Pa s}^n$ and a flow index of $n_{2\%w/v} = (1.7 \pm 0.5) \times 10^{-2}$, reflecting an extremely pseudoplastic behavior with a rapid viscosity drop under shear.

In contrast, the second sample has a higher $k_{3\%w/v} = 251 \pm 4 \text{ Pa s}^n$ and a slightly higher $n_{3\%w/v} = (9.7 \pm 0.8) \times 10^{-2}$, indicating a slightly more viscous system at low shear rates and a less pronounced shear-thinning response.

These differences again suggest that the 2% w/v sample has a more rigid, highly interconnected microgel network that resists initial flow but collapses rapidly under stress. The 3% w/v sample likely possesses more water in the interstitial spaces or weaker particle-particle interactions, as also shown in **Figure D.1**, leading to higher initial viscosity and a less drastic reduction under shear [4].

All these properties of mAlgs suspensions highlight an interesting potential future application. As a matter of fact, the 2% w/v suspension, might be more suitable for injectable or flowable systems due to its sharp viscosity drop, compared to the 3% w/v second sample offers a lower thixotropic behavior, a smaller moduli recovery and a higher shear stress needed to put in motion the suspension during injection. Overall, the 2% w/v mAlgs suspension is, therefore, the most promising solution not only for the 3D construct fabrication (see **Appendix D**), but also for a potential bioprinting application that could possibly automate the fabrication route.

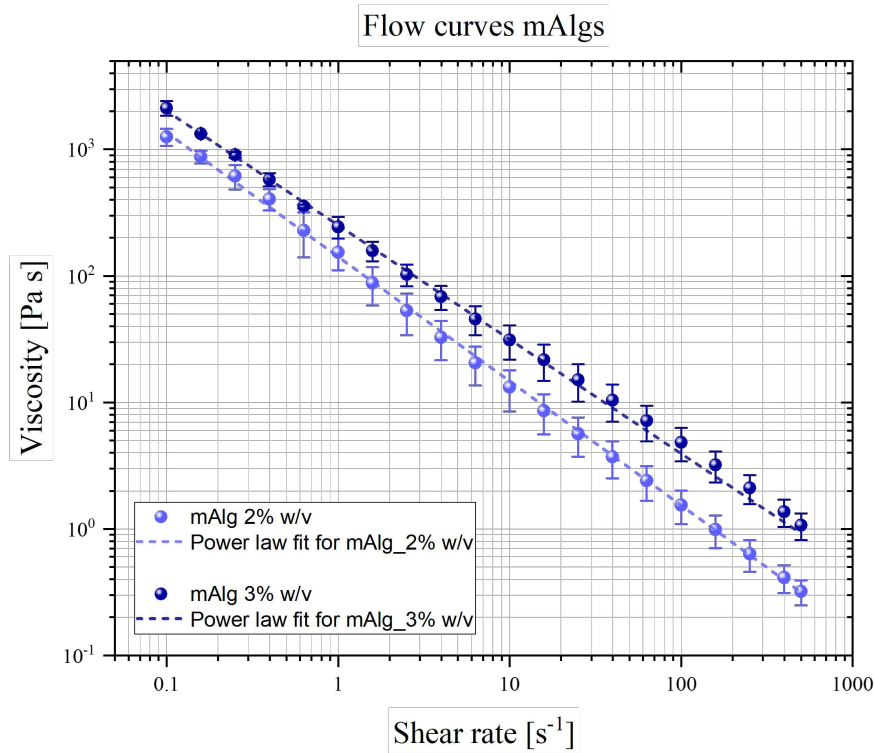


Figure D.4: Flow curve test on mAlg suspension prepared from 2% and 3% w/v alginate solutions, illustrating the relationship between shear stress and shear rate. This analysis demonstrates the shear-thinning behavior of the suspensions, characteristic of non-Newtonian fluids, as highlighted by the linear fit representative of a power law fluid.

Appendix E

Optimization of epithelial cells encapsulation in mAlgs

The encapsulation of A549 cells in alginate microspheres is highly sensitive to the concentration of $CaCl_2$ in the gelling solution, as calcium ions play a critical role in the crosslinking of alginate chains. However, it was experimentally noticed a certain difficulty in translating the synthetic procedure of blank mAlgs to the A549-loaded mAlgs.

As a matter of fact, a high concentration of 360 mM $CaCl_2$ ensures robust gel formation but on the other hand significantly compromises A549 cell viability due to the calcium ion gradient, which damages cell membranes.

Live/dead assay performed on A549/mAlgs obtained after 15 minutes of gelation in a 360 mM $CaCl_2$ 1:1 DMEM/PBS mixture shows how the vast majority of the epithelial cells are dead (**Figure E.1**). However, it can be seen how the synthesis allows the encapsulation of cells in a pretty well-shaped microspheres.

As a matter of fact, in a live/dead assay, the presence of predominantly red-stained cells indicates a high proportion of dead or membrane-compromised cells, as the red dye (e.g., Ethidium Homodimer-1) typically penetrates only damaged membranes. Nonetheless, it is also visible in some spots a certain degree of overlapping between green and red A549 signals. This

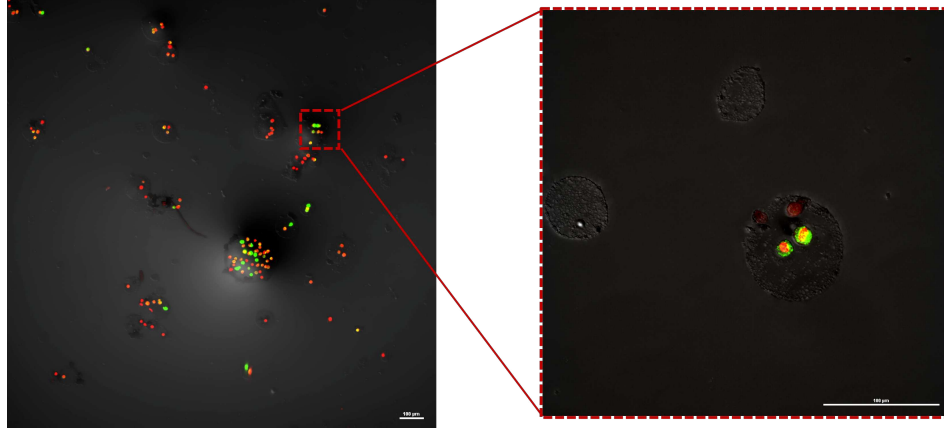


Figure E.1: Representative 4X and 10X images of **Live/dead** assay performed on A549-loaded mAlgs obtained after 15 minutes of gelation in a 360 mM $CaCl_2$. The high concentration of Ca^{2+} ions causes membrane disruption. Scale bar: 100nm

might suggest intermediate states, such as early apoptosis or compromised membrane integrity, potentially indicating transitional phases in cell viability [1].

Conversely, the absence of excess Ca^{2+} ions in the gelling solution preserves cell viability but fails to form stable mAlgs, resulting in dispersed cells.

As shown in **Figure E.2**, the A549 are almost completely viable, however they failed to assemble into mAlgs, due to the extremely low Ca^{2+} ions concentration in solution. All the nutrients were supplied thanks to the DMEM in the gelling solution, but since it was used a PBS 1X without $CaCl_2$ and $MgCl_2$, the overall concentration of ions was $[Ca^{2+}] \sim 1mM$.

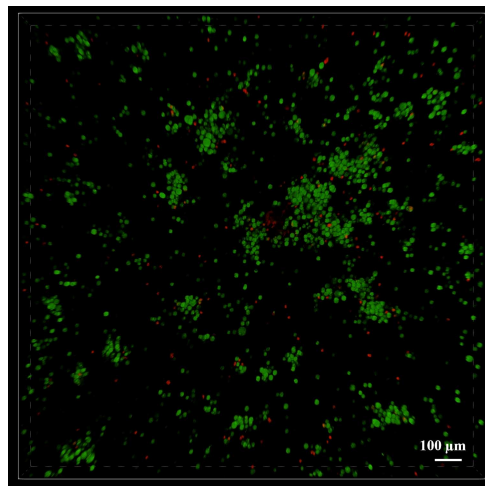


Figure E.2: Representative image of **Live/dead** assay performed on A549-loaded mAlgs obtained after 15 minutes of gelation in the plain DMEM/PBS 1:1 mixture. The low concentration of Ca^{2+} ions causes enables high cell viability, but is not enough to grant the actual formation of alginate microbeads. Scale bar: 100nm

Optimal conditions were achieved using 180 mM $CaCl_2$, which supported mAlg formation while maintaining high A549 viability. However, this concentration represents the limit for balancing cell compatibility and effective gelation, as it yields fewer mAlgs compared to 360 mM $CaCl_2$.

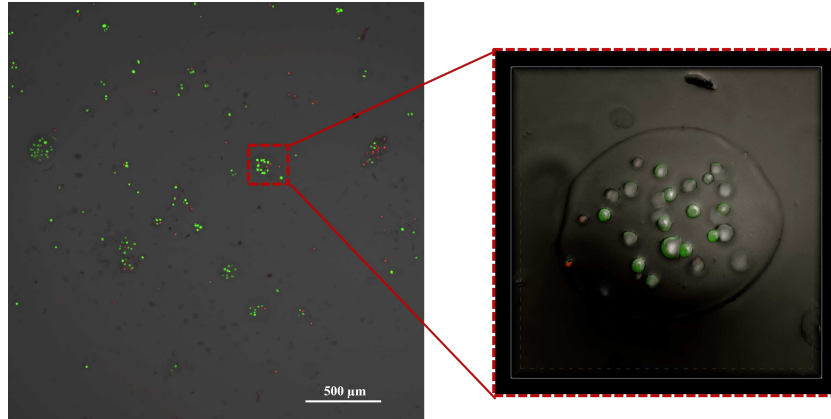


Figure E.3: Representative 4X and 20X images of Live/dead assay performed on A549-loaded mAlgs obtained after 15 minutes of gelation in a 180 mM $CaCl_2$. The concentration of Ca^{2+} ions allows viable mAlgs formation. Scale bar: 500nm

Additionally, the gelation kinetics at 180 mM are slower, and alginate dissolution in DMEM/PBS competes with gelation. Rheological analysis on 3D alginate pucks was therefore conducted to confirm the formation of an appropriate gel structure under these conditions.

In a frequency sweep analysis of 3D alginate gels crosslinked in a 1:1 DMEM and PBS solution with varying concentrations of $CaCl_2$, distinct differences were observed based on the initial alginate concentrations of 2% (**Figure E.4A**) and 3% w/v (**Figure E.4B**). At a low $CaCl_2$ concentration of 1 mM, no true gel formation was detected, with the moduli in the order of 10^{-6}) MPa.

However, starting from 73 mM $CaCl_2$, gel formation was evident for both alginate solutions. Notably, the gels derived from the 3% w/v alginate solution consistently exhibited higher moduli. The storage modulus (G') and loss modulus (G'') remained parallel to each other across the frequency range, indicating a consistent viscoelastic behavior, although both moduli displayed a non-zero slope.

These observations suggest that the concentration of $CaCl_2$ plays a critical role in the crosslinking density and mechanical properties of the alginate gels. The higher moduli in the 3%

w/v alginate solution can be attributed to the increased polymer concentration, which provides more binding sites for Ca^{2+} ions, leading to a denser and stronger gel network. The non-zero slope of G' and G'' indicates that the gels exhibit frequency-dependent viscoelastic properties, which could be due to the dynamic nature of the crosslinks within the gel matrix. This behavior is typical for physically crosslinked gels, where the network structure can rearrange under stress.

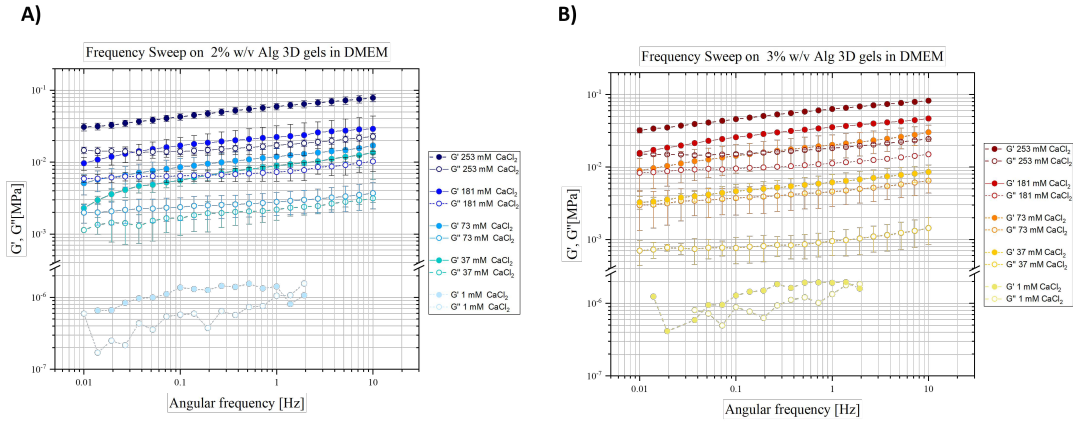


Figure E.4: Oscillatory rheological analysis of alginate 3D pucks starting from Alg solutions at different concentrations crosslinked in a DMEM/PBS 1:1 mixture at different $CaCl_2$ concentrations at $T=37^\circ C$. Frequency sweep analysis were run in the LVR region on 3D Alg pucks obtained from 2% w/v (A) and 3% w/v (B) alginate solution. Both storage (G') and loss (G'') moduli are reported as a function of oscillatory frequency.

When comparing the complex moduli of the same systems, it is observed that at high $CaCl_2$ concentrations of 253 mM, there is no significant difference between the moduli of gels formed from 2% and 3% w/v alginate solutions. However, at a lower concentration of 181 mM, the gels begin to behave differently: from this concentration downward, the moduli of the 3% alginate gels are consistently higher than those of the 2% gels at the same $CaCl_2$ concentration.

Furthermore, converting these complex modulus data into terms of mesh size reveals a decrease in the mesh size of the networks with increasing $CaCl_2$ concentration and with the increase in alginate concentration from 2% to 3%. Indeed, all experimental points, except those at 1 mM concentration, show a lower mesh size for the 3% samples.

These findings suggest that at higher $CaCl_2$ concentrations, the crosslinking density reaches a saturation point where additional alginate concentration does not significantly enhance the mechanical properties of the gel.

However, at lower $CaCl_2$ concentrations, the higher alginate concentration (i.e. 3%)

provides more binding sites for Ca^{2+} ions, resulting in a denser network with higher moduli.

The decrease in mesh size with increasing $CaCl_2$ concentration and alginate concentration indicates a tighter network structure, which is expected as more crosslinking points are formed. This tighter network in the 3% alginate gels -except at the lowest $CaCl_2$ concentration- results in stronger mechanical properties due to the increased number of crosslinks.

This behavior highlights the importance of both alginate and $CaCl_2$ concentrations in tuning the mechanical properties and network structure of alginate gels, also enabling to explain how the high $CaCl_2$ concentrations were toxic for the A549 cells. As a matter of fact, since the system reaches a sort of saturation in terms of possible crosslinking sites -for both Alg formulations- it means that an excess of free Ca^{2+} ions is present in solution, resulting in an aggressive behavior towards cells' membranes.

On the other hand, extremely low $CaCl_2$ concentrations do not allow gel formation or -at least- the formed gel is not strong enough to sustain a mechanical stress given by the magnetic stirring in solution. Furthermore, rheology still enabled to assess that also with this modified gelling solution, the 2% Alg solution is the best candidate for the formation of easily-dissolvable mAlgs, thanks to their smaller meshsize.

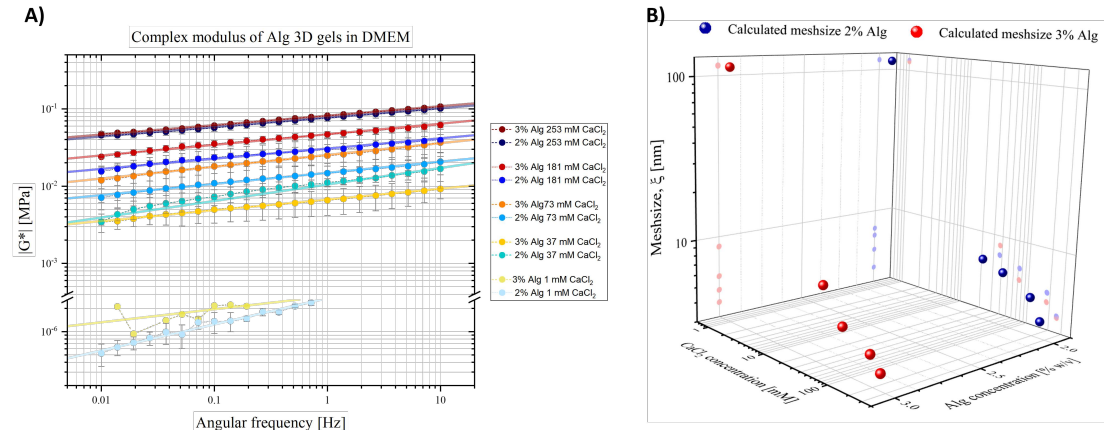


Figure E.5: Oscillatory rheological analysis of alginate 3D pucks starting from Alg solutions at different concentrations crosslinked in a DMEM/PBS 1:1 mixture at different $CaCl_2$ concentrations at $T=37^\circ\text{C}$. **(A)** Complex moduli as a function of oscillatory frequency obtained from frequency sweep data of 2% and 3% w/v. Linear fits are reported and from the intercept values the meshsize ξ was calculated and reported on a 3D graph in **(B)** for each sample and each $CaCl_2$ concentrations.

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Chapter 1 and Aim

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Chapter 2

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Chapter 3

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Chapter 4

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Appendix A

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Appendix B

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Appendix D

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