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3D-printed cell culture system as an *in vitro* platform for non-small cell lung cancer (NSCLC) modeling

By

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Declaration

I hereby declare that, the contents and organization of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

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I would like to dedicate this thesis to my beloved family

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Abstract

Lung cancers represent the first cause of cancer-related death worldwide. The most common subtype (i.e. lung adenocarcinoma) is characterized by high genomic instability, drug resistance and the insurgence of metastasis in distal organs. Unfortunately, the designing of *in vitro* systems that fully resemble the *in vivo* features fails, due to the intra- and inter- tumor heterogeneity and to the presence of a rich microenvironment, that cooperates with tumor cells during cancer progression to create a pervasive matrix. For this reason, over the years, animal models have been used to test drug efficacy and mechanisms that lead to therapy resistance. Nonetheless, the cost, the differences with the human system and the ethical concerns lead to the development of new and more efficient *in vitro* models which could replace the *in vivo* ones. For this reason, the aim of this project is to design a 3D *in vitro* system of lung adenocarcinoma to be used for the study of tumor cellular and molecular features and drug responses. In the first part of the work, the development of a matrix which could support the growth of the tumor model has been described. In particular, methacrylated gelatin (GelMA) has been selected for its biocompatibility, the presence of cell-adhesive motifs and printability. Two different models of lung adenocarcinoma have been identified according to the tumor cells used, i.e. A549 and H1299 with the fibroblasts (i.e. MRC-5 cells) and their response to the new matrices has been analyzed. Further, the feasibility of promote spheroids formation inside 3D printed GelMA scaffold has been demonstrated. As reported in the literature, vasculature plays a crucial role in nutrient supply and in tumor progression. An effective approach to introduce this aspect in the models could be the extrusion-based 3D-bioprinting, by using sacrificial inks, which allow to obtain empty channels inside the scaffold. In this view, printability of Carbopol, a copolymer of acrylic acid usually used in topic drug formulation and noteworthy for its thixotropic properties, has been investigated. Carbopol can be easy remove, thanks to buffer dilution and the endothelial component could be seeded onto the empty channels. In the last part of the work, the design of a microfluidic platform has been described. Recent studies demonstrated that dynamic culture increases the reliability of the response of the cells to drug treatment. In this work, digital light processing (DLP) based printing has been selected to test the printability of several acrylate resins, thanks to its fast printing speed and high resolution. PEGDA and acrylate PDMS resulted to be the best candidates for the development of a microfluidic device, for the growth and development of lung adenocarcinoma 3D *in vitro* models and drug testing.

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Chapter 1

Introduction

1.1 Respiratory system: general overview

In the next paragraphs, the main elements of the respiratory system will be dissected to show the complexity and the heterogeneity of this organ. Further, a brief overview of the main diseases that impair the respiratory function will be discussed to understand the severity of those pathologies and the urgent need for reliable *in vitro* models to accelerate the development of efficient therapies.

1.1.1 Respiratory system organization

Mammals' respiratory system is divided into two distinct parts characterized by different localization, function, and structure. The upper tract starts from the nose and ends at the larynx, while the lower one consists in the trachea and lungs (i.e. bronchi and alveoli). Importantly, the latter are involved in gas exchange with the pulmonary vasculature ¹.

The lower fraction is a heterogeneous organ and it is characterized by several cellular and acellular components that cooperate to maintain respiratory function. Its epithelium organization is summarized in Fig.1.1. Specifically, the airways are mainly composed by a pseudostratified epithelium, which contains several cell lineages including ciliated cells, mucus-secreting goblet cells and basal and club cells, which are involved in tissue renewal. In addition to the maintenance of the respiratory function, this strongly interconnected epithelium represents a mechanical barrier that blocks pathogens diffusion and preserves tissue integrity. Moreover, various mesenchymal cell types, including smooth muscle fibroblasts and pericytes support organ homeostasis and functionality ².

In the distal part the alveoli, which are highly vascularized sacs are committed to controlling the gas exchange. Alveoli mainly consist of two epithelial cell populations, called alveolar type I (AT1) and AT2, also named pneumocytes. These cell lineages differ both in structure and role in the lungs' physiology. Indeed, the most abundant AT1s, which show a flattened and squamous appearance, mediate gas exchange and are strongly associated with the pulmonary capillary. Instead, the cuboidal AT2s produce the mucus, that reduces surface tension and avoids alveoli collapse. Interestingly, also alveolar epithelium is characterized by slow renewal, in particular, the activation of stem cell function in AT2 produces clonal foci, which are involved in the production of AT1 cells ^{3,4}. Importantly, the space between the endothelial cells of the vessels and AT1 cells, called the pulmonary interstitium, contains fibroblasts and collagen fibers that confer elasticity to the tissue. Further, the lungs are enveloped by the pleura, which consists of two layers separated by a thin amount of fluid, that contains

mesothelial cells and mucopolysaccharides. It acts as a lubricant for the smooth movements that happen during respiration ⁵.

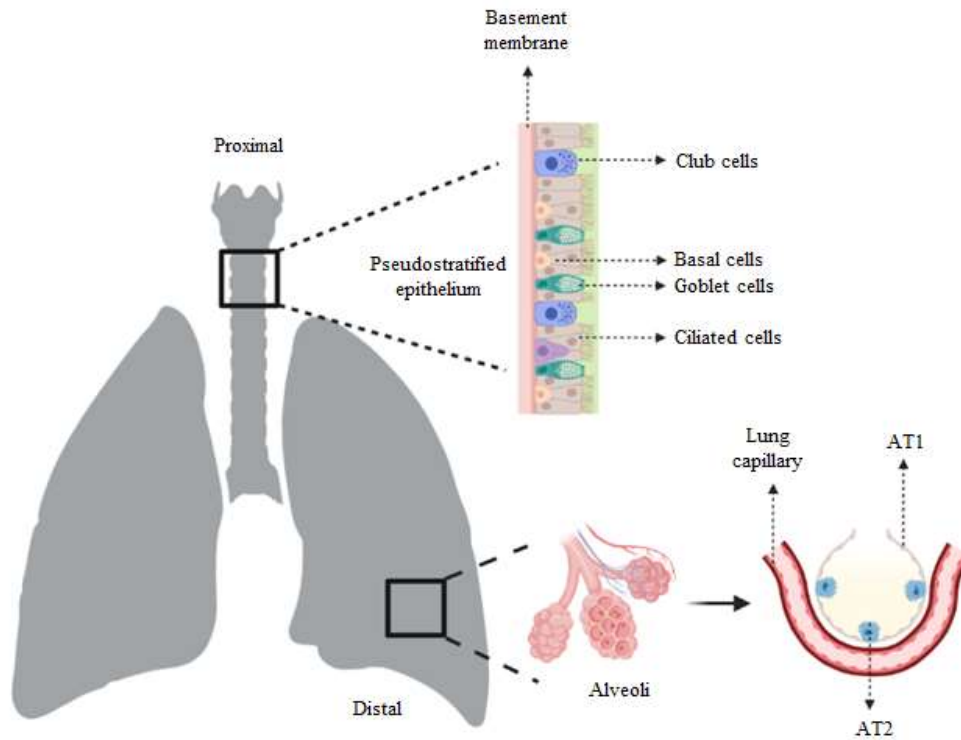


Fig. 1.1: Schematic representation of lung epithelia. Specifically, components of the pseudostratified epithelium in the proximal area of the lungs (i.e., ciliated, club, basal and goblet cells) and of the alveolar one in the distal area (i.e., AT1 and AT2) are shown. Created with BioRender.

The respiratory tract is protected from pathogens and noxious compounds by the mechanical barrier provided by the pseudostratified epithelium and the mucus, and by the activation of the immune responses. Mucus consists of water, polysaccharides, and proteins whose main components are mucins, a high-molecular weight glycoprotein family. They could be membrane-bound (e.g., MUC1) or secreted (e.g., MUC5) and are involved in the maintenance of viscoelastic properties of the mucus. The uncontrolled release of these components strongly impact tissue functionality and is associated with asthma and chronic obstructive pulmonary disease (COPD) ^{6,7}. As better described in Fig. 1.2, pathogens, like bacteria or viruses, are actively recognized by sensor cells (i.e., airway epithelial cells, alveolar macrophages, and dendritic cells), which activates innate immune response by secreting several factors (i.e., interferons and cytokines) and attracts T cells and Natural Killer cells (NKs). Their role is the clearance and, eventually, the repairing of the tissue, indeed, type II immunity is activated when the organ is invaded by multicellular parasites (e.g. helminths) or by allergens or venoms and it is mediated by mast cells ⁸.

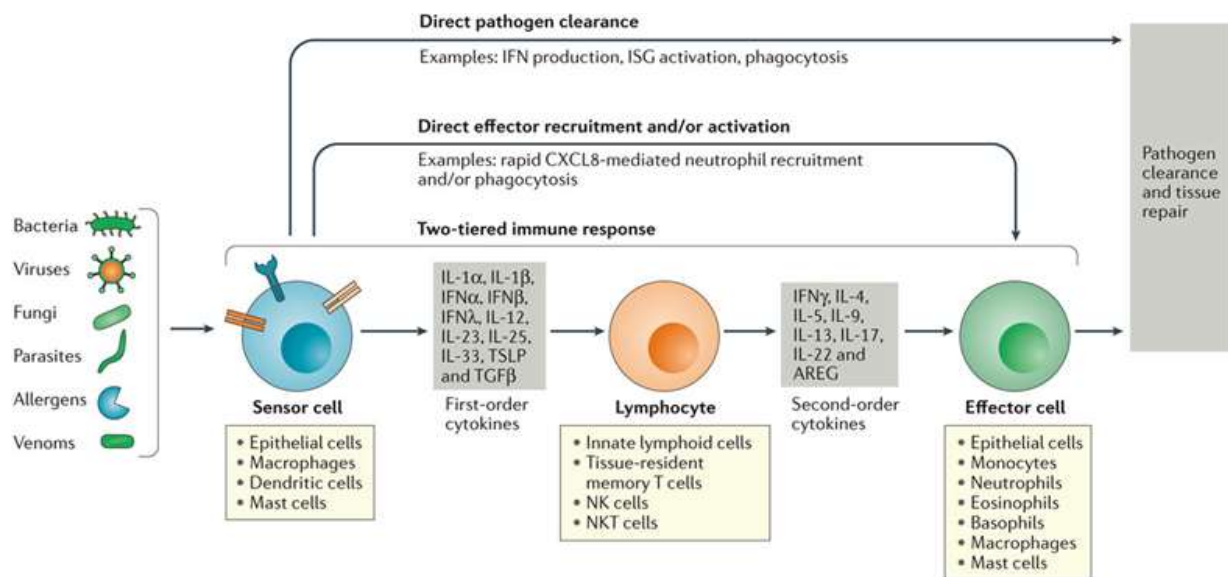


Fig. 1.2: Schematic representation of the activation steps of the immune response in health lung tissue in the presence of various pathogens. Reproduce with permission from Iwasaki et al., 2016 ⁸.

1.1.2 Lung diseases

Since their heterogeneous structure, the respiratory system can be affected by several illnesses characterized by a plethora of symptoms and consequences on its function. For instance, asthma and COPD can affect the airways and, although they are characterized by similar symptoms, the causes of the inflammation are different.

Asthma is induced by exposure to allergens (e.g. plant or animal proteins, metals, chemical compounds) and it is mediated by IgE and eosinophils. It is mainly characterized by bronchoconstriction, because of the activation of the smooth muscles and the thickening of the basal membrane.

COPD, instead, is usually caused by tobacco and pollution and it is associated with airways obstruction. It represents a leading cause of death worldwide and the chronic and progressive inflammation induced by neutrophils and macrophages is often associated with comorbidities ^{9,10}.

Lungs can be also affected by genetic disorders, like cystic fibrosis. Cystic Fibrosis is an autosomal recessive pathology correlated to mutations in cystic fibrosis transmembrane conductance regulator (CFTR) gene. Its severity is linked to the molecular defects caused by the mutation (i.e., impaired functionality or decreased number of CFTR). CFTR is a chloride channel, which transports ions across the apical membrane of epithelial cells, in addition, it plays an important role also in bicarbonate secretion and in the inhibition of sodium transport. Cystic fibrosis has a systemic effect, especially on the lungs, pancreas and gastrointestinal tracts. For instance, the impaired ions exchange and pH regulation lead to the accumulation of mucus in the airways, resulting in a decreased pulmonary capacity ¹¹.

Among the infections of the lower tract of the respiratory system, pneumonia affects 489 million people globally and it is associated with high mortality in children and older people, especially in the poorest areas of the world. Many microorganisms (e.g. bacteria and viruses) can cause pneumonia, which is worsened by comorbidities like COPD, asthma and cancer ¹².

The respiratory capacity can also be impaired by diseases that affect the interstitium, including some types of pneumonia, edema and fibrosis. For instance, idiopathic pulmonary fibrosis is characterized by an aberrant activation of the epithelium of the alveoli and the transition of resident fibroblasts into myofibroblasts, which strongly produce extracellular matrix (ECM) components. All these modifications of lung architecture decreased the capability of gas exchange with important effects on patient life quality ¹³.

1.1.3 Lung cancer

Lung cancer is a heterogeneous disease and represents the leading cause of cancer-related death worldwide in both sexes ¹⁴. Lung cancer is classified into two main groups, according to the cells of origin and the histological appearance: (I) small-cell lung cancer (SCLC) and (II) non-small-cell lung cancer (NSCLC) (Fig. 1.3).

(I) SCLC, represents 15% of the total diagnosis and it is strongly associated with tobacco consumption. It mainly originates from neuroendocrine lung epithelial cells and it is associated with a low survival rate ¹⁵. Interestingly, its insurgence is driven by the inactivation of two important tumor suppressors: p53 and retinoblastoma (Rb) ¹⁶. Several therapeutic approaches are selected to reduce mortality. The most used are chemotherapy and radiotherapy, whereas surgery is the less effective one and it is not recommended for higher than stage I patients. Recent clinical studies are trying to improve the effectiveness of the treatment by testing immunotherapy, which has already shown promising results for NSCLCs ¹⁷.

(II) The second group, NSCLC, represents 85% of the total lung cancer diagnosis and it is classified according to pathological characteristics. The most abundant phenotypes are adenocarcinoma (ADC) which arises in the distal portion of the lung (i.e., from AT1 and AT2) and squamous cell carcinoma (SCC) which grows in the proximal area. The last group is the large-cell carcinoma which is genetically and morphologically distinct from ADC and SCS ¹⁸. These subtypes could be distinguished by their molecular signature, which is influenced by the mutations carried by the tumor cells and their localization ¹⁹. Unfortunately, they are characterized by huge genomic instability and high intratumor heterogeneity ²⁰, for instance, driven mutations in ADC occur in KRAS, epidermal growth factor receptor (EGFR) and BRAF but during progression the signature changes ²¹. The election treatment is the resection of the tumor, followed by a chemotherapy cycle to prevent recurrence and metastasis development. Radiotherapy is a recommended approach for patients in which cancer could not be surgically removed. Later-stage carcinoma can also be treated by using targeted therapies, according to histological cell type and genomic mutation profile. Unfortunately, the life expectancy of patients with advanced-stage tumor is still poor ²².

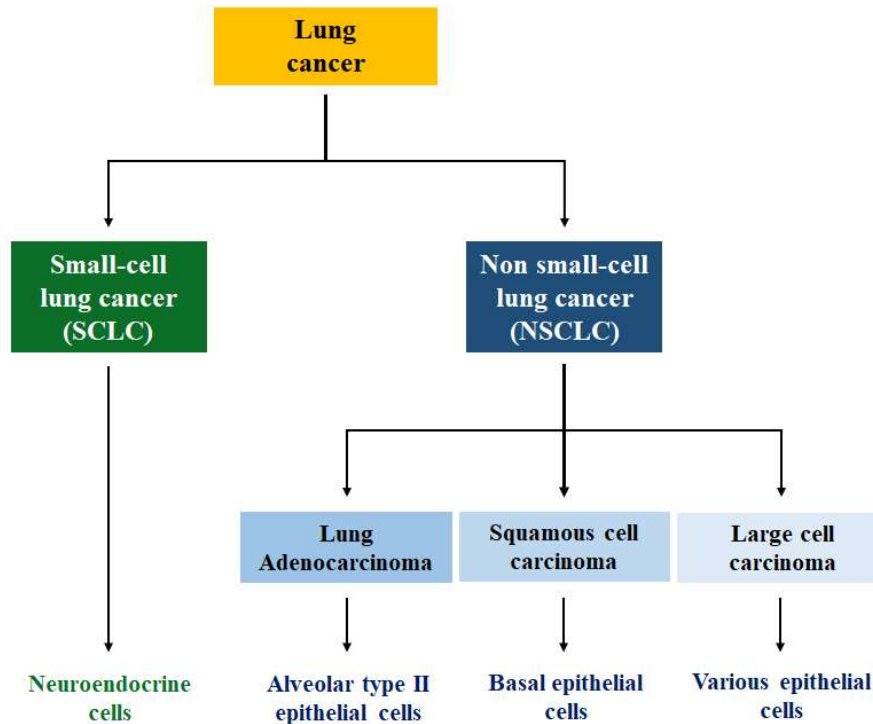


Fig. 1.3: Schematic classification of lung cancer subtypes.

1.2 Tumor microenvironment and its role during cancer progression

NSCLC is a complex disease characterized by different genetic mutations and a wide genomic instability. As shown in Fig. 1.4, cancer progression involved several players that together create a heterogeneous microenvironment. In this setting, several cell types and matrix components interact to promote tumor growth, invasion and migration¹⁸. In the next paragraphs, the main elements of the stroma and the ECM will be described and their role in tumor progression will be dissected. Indeed, as described below, the microenvironment is crucial for several aspects, as the growth and the aggressiveness of the tumor, the development of effective therapies and the insurgence of drug resistance. Hence, its study is essential for understanding the way in which lung cancer developed and finding new therapeutic targets.

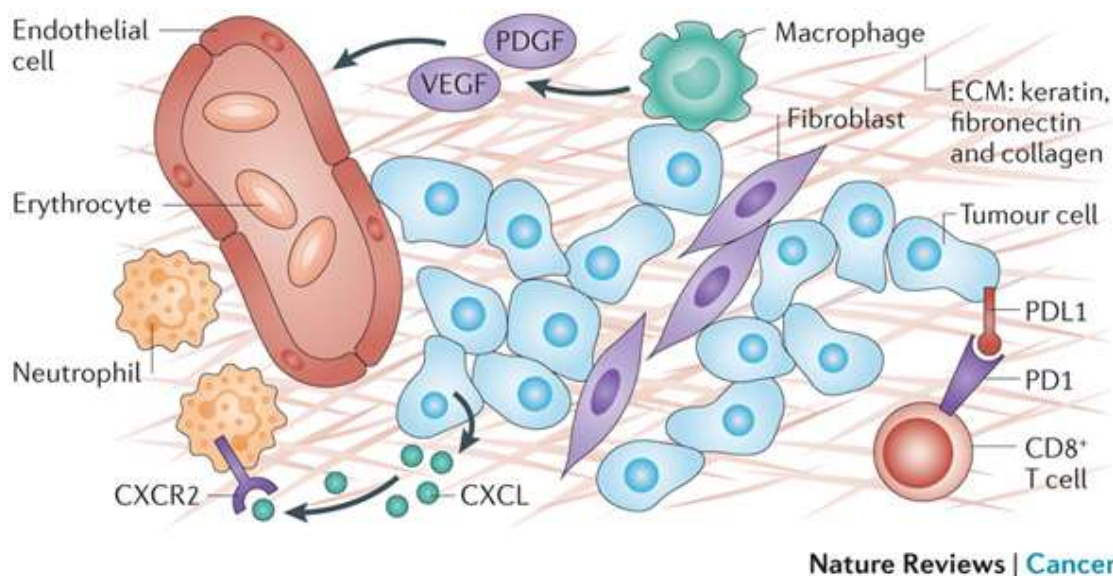


Fig. 1.4: The heterogeneous tumor microenvironment consists in several cell types: tumor cells, recruited endothelial cells, fibroblasts, and immune cells (i.e., neutrophil, macrophages, T cell). The extracellular matrix (ECM) which includes keratin, fibronectin, and various collagen types, provides support to cancer growth. Reproduce with permission from Chen et al., 2014 ¹⁸.

1.2.1 Extracellular matrix components and their role in lung cancer

The ECM is a three-dimensional structure responsible for the architecture, organization and homeostasis of tissues and organs. For instance, the basement membrane provides support to epithelial cells, while the interstitial matrix gives nourishment. In general, ECM regulates cell growth, survival, motility and homeostasis of the cells that formed the tissues ²³.

Many elements are present in the ECM, namely collagens, proteoglycans, hyaluronan, fibronectin, laminin and elastin, as well as the integrin ECM receptors (Fig. 1.5). One of the most abundant and ubiquitous are collagens. Their role is the maintenance of tissue integrity, however, remarkable differences can be found among the components of collagen family. Indeed, fibrillar and globular collagens differ in structure and in function, as an example Collagen type I and II form fibrils that in turn regulate the structure and the stiffness of the matrix ²⁴.

Another important component is fibronectin, a glycoprotein that regulates many cell behaviors including differentiation and epithelial-mesenchymal transition. Further, it interacts with collagens, growth factors and integrins on cells' surface promoting the activation of specific intracellular signaling pathways ²⁵.

Other players are laminin and tenascin families, proteoglycans and metalloproteinase (MMP) family, whose role is the remodeling of the surrounding matrix ²⁶.

Since their importance in tissue homeostasis, their dysregulation leads to the insurgence of disease with increasing degrees of severity. Interestingly, ECM in tumor plays an important role during every stage of

progression. Indeed, its modification can promote cell proliferation and survival, angiogenesis, and, in the end, migration, invasion and metastasis formation ²⁷. Moreover, changes in matrix stiffness strongly influence cell behavior and cancer progression ²⁸.

Healthy lung ECM is mainly composed of collagens (type I, II, III, V, XI) and glycoproteins, such as fibrillin. Specifically, the alveolar interstitial part consists mostly of collagens type I and III, fibronectin and elastin. However, as mentioned before, during cancer progression, many modification occurs, including the increase of fibrillar collagens, hyaluronic acid, tenascin C and fibronectin amount ²⁹. In adenocarcinoma, diffuse fibrosis caused by the deposition of collagen-containing hydroxylated prolines is common ³⁰. Importantly, stiffness increase strongly influences several cell behaviors, including growth ³¹, migration ^{32,33} and the epithelial-to-mesenchymal transition (EMT) ³⁴.

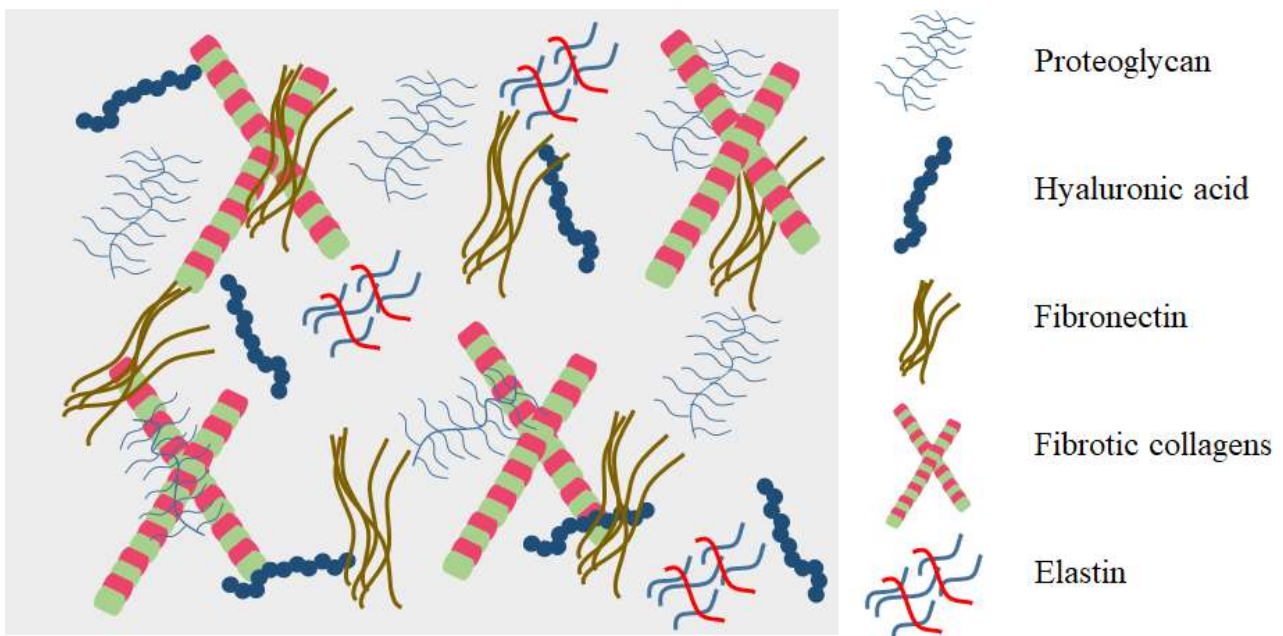


Fig. 1.5: Schematic representation of ECM components

1.2.2 Fibroblasts activation and their role in cancer progression

In the tissue, ECM production is mainly due to fibroblasts. Fibroblasts' most well-known biological role is the production and secretion of all components of the ECM. Hence, they create a supportive network composed of fibrillar proteins, and they maintain the homeostasis. Further, they produce the adhesive proteins (i.e., fibronectin and laminins) that anchor cells to the matrix. In addition, they secrete enzymes (e.g. MMPs), that digest the ECM components and allow the modification of the microenvironment ³⁵. Importantly, dormant fibroblasts can be activated by several stimuli (e.g. stress, wound, inflammation) to produce and remodel the surrounding matrices and, eventually, repair the tissue. Activated fibroblasts, called also myofibroblasts, strongly increased the

expression of α -smooth muscle actin (α SMA; also known as ACTA2), a cytoskeletal protein associated with smooth muscle cells, that could be used as a marker for these types of fibroblasts ³⁶.

In the lungs, alveolar fibroblasts modulate the ECM, providing support to the epithelium and the endothelium whose proliferation and differentiation are strongly correlated to the cross-talk with the components of the matrices. Further, the renewal of AT2 and their differentiation into AT1 are controlled by the secretome of these cells ^{1,37}.

Since their importance in lung homeostasis, fibroblasts dysregulation leads to disease insurgence. For instance, in idiopathic pulmonary fibrosis, lung epithelium acquired several alterations which, in turn, lead to the abnormal activation of resident myofibroblasts. These modifications promote the deposition of fibrotic matrices, which impaired the respiratory capability of the lung ³⁸. In COPD, the reduced proficiency of fibroblasts to deposit elastic fibers strongly impaired the normal functionality of the organ ³⁹. In NSCLCs, cancer-associated fibroblasts (CAFs) play a crucial role in the progression of the disease. Since the fibrotic nature of the disease, cross-talk between the components of the tumor microenvironment is necessary for the invasion of the tissue. As summarized in Fig. 1.6, fibroblasts support metabolic reprogramming, angiogenesis, proliferation and creation of a pervasive microenvironment ^{40,41}. Importantly, the education of the resident fibroblasts is promoted by the tumor, indeed, gene expression analysis revealed a significant modulation in genes involved in angiogenesis, matrix degradation, cell activation and proliferation in lung fibroblasts, when cultured with tumor cells ⁴². Therapeutic intervention usually fails, because of the insurgence of drug resistance, although an important role is played also by the CAFs. Indeed, the main mechanisms, in which CAFs play a crucial role, are linked to the ability of the tumor cells to avoid apoptosis, to easily repair DNA damage, to modify the targeted molecules and to enhance drug efflux ⁴³. Moreover, ECM remodeling, epithelial-to-mesenchymal transition and metabolic reprogramming of the tumor cells induced by the resident fibroblasts, strongly decrease drug efficacy ⁴⁴.

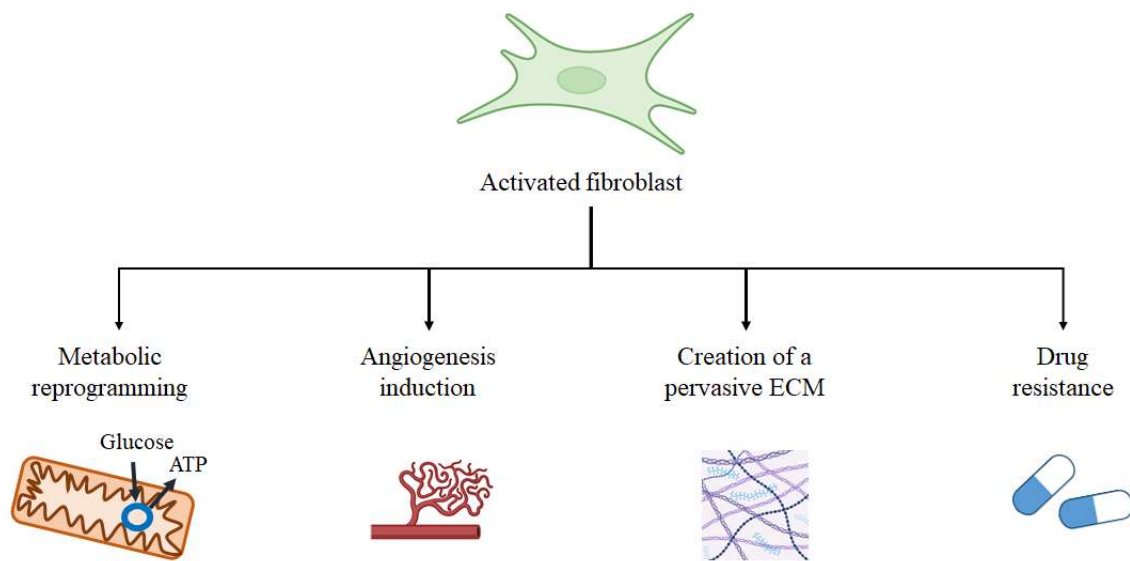


Fig. 1.6: Schematic representation of the role of activated fibroblasts in the tumor microenvironment. Created by BioRender.

1.2.3 Immune cells

Another important player in tumor progression is the immune system. Indeed, although an immune response against a tumor could be promoted by cytotoxic T cells, natural killer (NK) cells and a subtype of macrophages, regulatory T cells and myeloid-derived suppressor cells (MDSCs) prevent the response and promote metastasis formation ⁴⁵. For instance, lung cancer presents a high level of MDSCs, which in turn block the activation of effector T-cells and NK cells and promote the differentiation of regulatory T cells ⁴⁶. Since the immune response is strongly compromised in NSCLCs, immunotherapy can represent a new therapeutic approach to improve patient survival. Several techniques have been studied: vaccines to activate immune effector cells, stimulation strategies including cytokines, adoptive cell therapy, oncolytic viruses and antibodies against immune checkpoint molecules to neutralize immunosuppressive mechanisms ⁴⁷.

1.2.4 Angiogenesis

The formation of new vessels or angiogenesis is necessary to sustain the growth of the tumor, hence, during cancer progression, the activation of mediators of angiogenesis is enhanced. Among those, the most studied are Vascular Endothelial Growth Factor family (VEGF), Transforming Growth Factor (TGF), Tumor Necrosis Factor- α (TNF- α), Plated-Delivered endothelial Growth Factor (PDGF) and several cytokines. At the same time, many inhibitors of vessels formation are downregulated ⁴⁸.

Thus, angiogenesis plays a crucial role also in the development of lung cancers. For instance, Wnt5a, a regulator of the Wnt pathway which is implied in tumorigenesis, is found overexpressed in NSCLCs and this is correlated to higher microvessels density ⁴⁹. Anti-angiogenic therapies based on antibodies are used for the treatment of NSCLCs. Unfortunately, the outcomes are not as promising as expected, because of the insurgence of resistance. The main causes are the ability of tumor cells to form vessels in an angiogenic-independent way (i.e. tumor cells that act as endothelial cells or attract vessels from adjacent sites), the activation of compensatory pathways to continue to promote angiogenesis and the activation of the tumor microenvironment (e.g. stromal cells and immune cells) to block or avoid the effects of the therapies ^{50,51}.

1.3 Matrix stiffness in cancer: mechanisms and biological effects

Tumor, stromal, endothelial and immune cells are surrounded by a complex matrix, and as described in the previous paragraphs, it is able to modulate cancer growth and progression. Another player that strongly influences many aspects of disease development, consists in the mechanical properties of the microenvironment. Indeed, they are able to modify the behavior of cancer cells by changing proliferation rate, invasion capability, vessel formation, nutrients availability and responses to drug treatments (Fig. 1.7) ⁵². This section will describe how stiffness changes in tumor microenvironment, the molecular pathway involved in this process and the effects of the modulation of cell behavior.

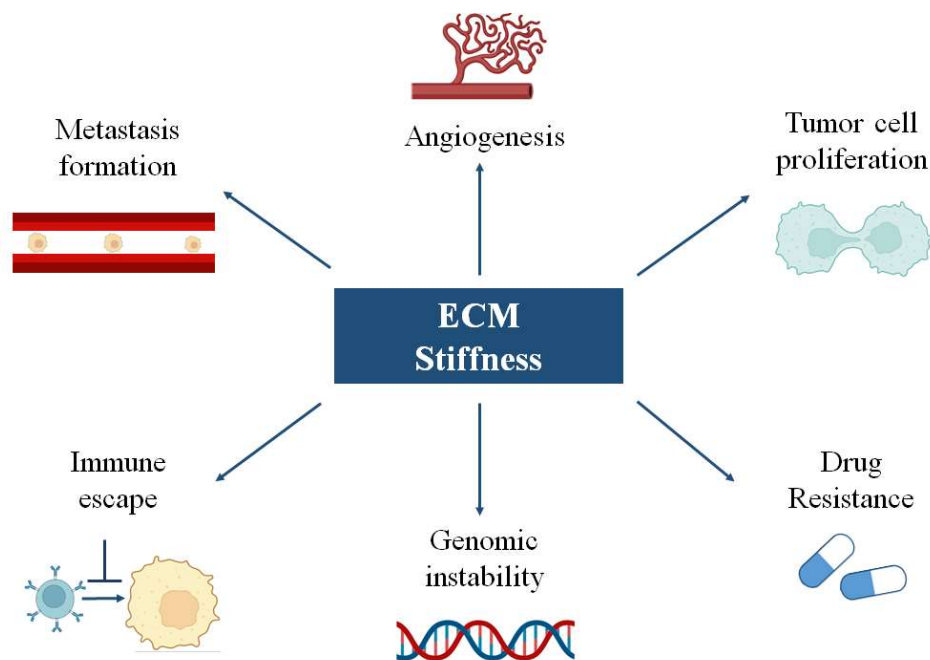


Fig. 1.7: Schematic representation of the effect induced by ECM stiffness modulation. Created by BioRender.

1.3.1 Mechanisms involved in matrix stiffening

During cancer progression, ECM composition and production is strongly regulated by tumor cells and the surrounding stroma. Importantly, these modification leads to an increase in tumor stiffness promoted by matrix deposition, contraction and crosslinking ⁵³. Indeed, fibrosis is usually present in solid tumors, and it is caused by the deposition of several ECM components. The main players are: the fibrillar type I collagen whose production is induced by profibrotic cytokines, like TGF- β 1, and fibronectin which triggers cell adhesion and invasion ⁵⁴. Interestingly, upregulation of type I collagen is found in lung cancers tissue and it is correlated to poorer prognosis ⁵⁵, whereas, fibronectin overexpression is linked to cell growth, migration, invasion and chemoresistance ⁵⁶.

Another mechanism involved in tissue stiffening is the cross-linking. For instance, collagen cross-linking is strongly involved in the insurgence of lung fibrosis in several diseases. This process is mediated by Lysyl oxidase (LOX) family which contains a large group of copper-dependent enzymes and they promote fibril stabilization, organization and size homogeneity ⁵⁷. Interestingly, LOX expression is enhanced in lung adenocarcinoma, promoting cell invasion and proliferation ⁵⁸, indeed they lead to metastasis formation by creating a fibrous and pervasive environment ⁵⁹.

1.3.2 Mechanosensing in response to mechanical stimuli

In all tissues, cells are continuously exposed to the mechanical stimuli induced by the surrounding matrices and their dynamical response modulates their behavior and modifies the microenvironment. Indeed, it has been shown that tissue stiffness is profoundly correlated with their development, homeostasis, risk of disease and response to therapeutic treatments ⁶⁰. Variation in ECM stiffness is observed in many diseases, including lung fibrosis and cancers and it is promoted by the activation of several intracellular pathways ⁶¹.

Hence, mechanosensing acquires huge importance in the determination of cell fate and its studying is crucial to understand how diseases progress and to develop efficient therapeutic treatments. Indeed, matrix elasticity has an impact on cell function, stem cell differentiation and on the activation of various intracellular pathways ⁶². Importantly, modulation of the microenvironment stiffness results in changes in the conformation of membrane components, such as vinculin, integrins and focal adhesion kinase (FAK), which are involved in cell adhesion to the surrounding matrix. Those changes usually result in the activation of intracellular signaling pathways, which in turn modulate cellular behavior. For instance, stiffer matrices promote the translocation into the nuclei of two transcriptional coactivators called yes-associated protein 1 (YAP) and PTZ-binding motif (TAZ), which are players in the activation of genes involved in the regulation of cell cycle and proliferation rate, metabolic activity and in cell differentiation (Fig. 1.8) ⁶³. Since YAP and TAZ activate the expression of genes involved in cell proliferation, migration and invasiveness, their activation is positively modulated in lung cancer. Indeed, matrix stiffening promotes the nuclear translocation of these transcriptional factors which in turn promotes survival, progression, metastasis formation and drug resistance ⁶⁴. Further, also stem cell commitment is linked to

cytoskeleton activation mediated by Rho/Rho-associated protein kinase (ROCK) pathway which in turn is activated in response to mechanical stimuli ⁶⁵. Importantly, ROCK signaling is significantly upregulated also in lung cancer and it is associated to tumor growth, invasion, and angiogenesis promotion. Indeed, the increasing stiffness of the tissue leads to a higher activation of Rho and ROCK which in turn positively modulates cell contractibility. Moreover, the upregulation of this pathway increases the proliferation and the migration of lung cancer cells, but decreases apoptotic commitment (Fig. 1.8) ⁶⁶.

Mechanosensing has an important role also in cell populations surrounded by variable matrix composition, e.g., endothelial cells. They are subjected to the shear stress induced by the blood flow and to the interaction with the connective tissue and respond through the activation of ion channels, G-protein and G-protein coupled receptors, integrins and intracellular junction protein ⁶⁷.

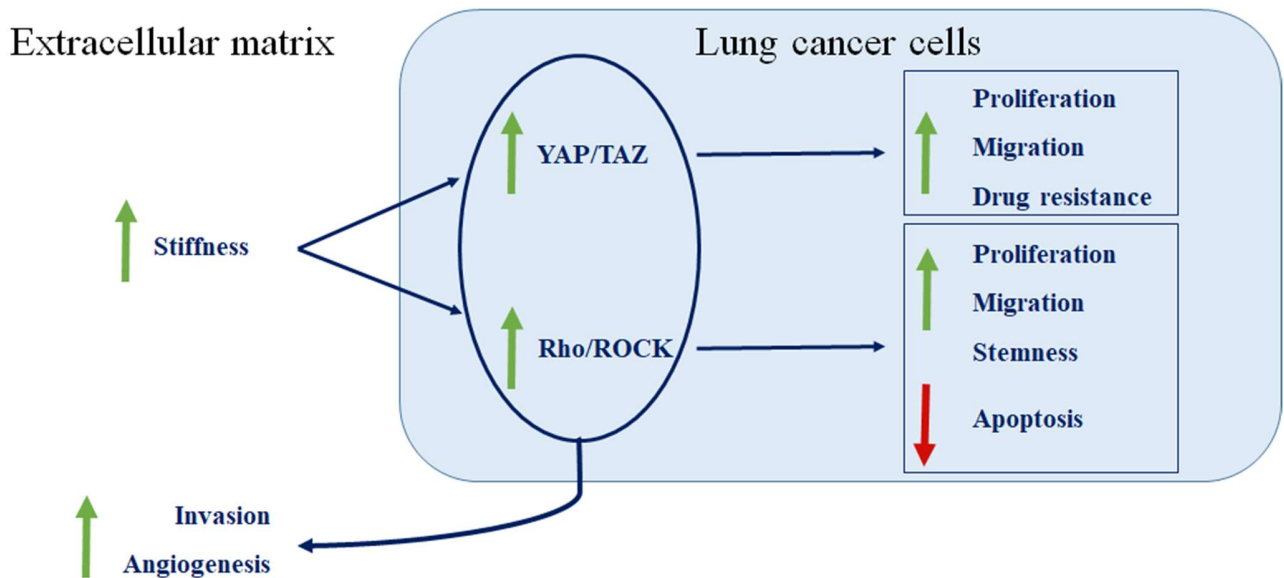


Fig. 1.8: Schematic representation of the activation of YAP/TAZ and Rho/ROCK intracellular pathways in response to matrix stiffness increase.

1.4 Models for cancer studying: general overview

As shown in the previous paragraphs, lung cancer is a heterogeneous, complex and severe disease. The role of the players involved in the failure of the treatments must be dissected to improve drug efficiency and patient's survival rate. To do this, in the years, several *in vivo* and *in vitro* models have been optimized (Fig. 1.9). In these paragraphs, the main advantages, and drawbacks of each one will be investigated.

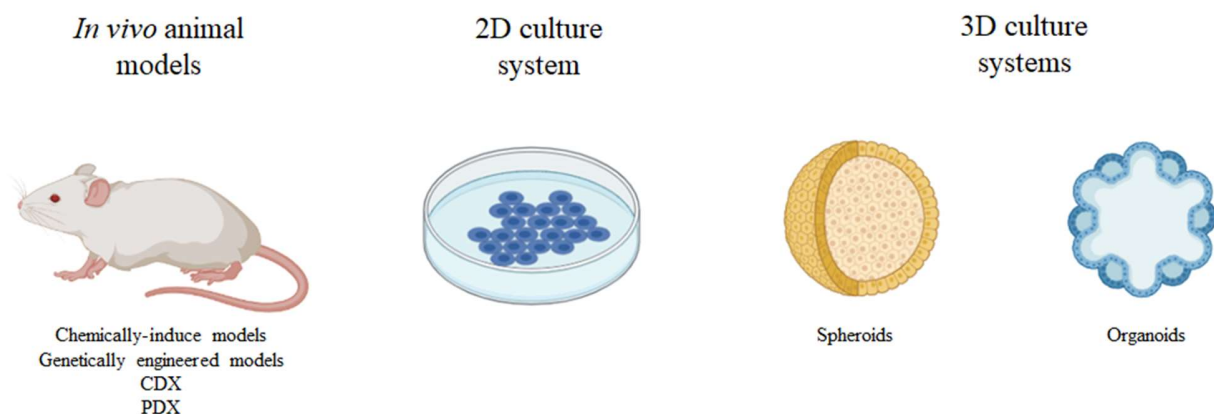


Fig. 1.9: Schematic representation of the main *in vivo* and *in vitro* strategies for the studying of lung cancer. Created by BioRender.

1.4.1 *In vivo* animal models

Animal models are widely employed to study human diseases including cancer. They help in the unrevealing of gene mutations that occur during tumor progression and the development of effective drug treatments. Mice are the most used thanks to their genome being close to the human one. Several methods to obtain cancer mouse models have been optimized over the years and include chemically induced model (e.g. carcinogens administration), cell line-derived xenograft (CDX), patient-derived xenograft (PDX) and genetically engineered mouse (e.g. induction of oncogene expression or deletion of tumor suppressor genes) ⁶⁸.

Since the large diffusion and the low survival rate, mice that carry lung cancer have been studied. For resembling NSCLC features, several mutations have been introduced in several genes including in KRAS (e.g., for adenocarcinoma model), BRAF, EGFR (e.g. for bronchioalveolar carcinoma model), Lkb1, Rac1, NFkB, and p53 loci. Models of SCLC have been developed as well, and as described in the previous paragraph, the predominant modifications in this subtype are the loss-of-function mutations in Rb and p53 loci. Hence, mouse models carrying these inactivated genes are used for the analysis of this rare disease ⁶⁹.

Ethical concerns, costs, and the limitation in fully resemble human features arise questions about the substitution of animal models. In 1959, Russell and Burch defined three R principles (i.e. 3Rs) to change the use of animal models⁷⁰:

- 1) Replacement: the substitution of living animals when *in vitro* alternatives are available.
- 2) Reduction: use of the minimum number of animals to obtain meaningful data and to achieve project goal.
- 3) Refinement: improvement of animal conditions to reduce pain, stress and suffer during the experiments.

Starting from the early 1980s, several legislations began to regulate the use of animal models. Importantly, in 2010 the European community enacted the European Directive 2010/63/EU, which provides a solid basis for the application of the 3Rs principles in animal experiments ⁷¹. For this reason, great interest in the development of *in vitro* systems which could totally or partially substitute animal models have arisen.

1.4.2 2D *in-vitro* culture systems

The classic *in vitro* system for cell analysis consisted of traditional 2D culture, in which cells are grown as a monolayer attached to a plastic surface. The main advantages are that the culture can be easily handled, the low-cost of its maintenance and the availability of optimized assay for cell analysis. However, this approach shows a huge limitation, which is the lack of all the stimuli that regulate cell proliferation, fate, differentiation and interactions derived from the surrounding environment in a normal tissue ⁷². For instance, in 2D culture the natural shape of the cells is lost and the junction between them does not fully reflect the one found *in vivo*. Then, proliferation rate and differentiation are strongly influenced leading to misunderstanding the real pathway and events that occur in physiological or pathological conditions. Further, also the screening of new drugs and their efficacy is not completely reliable in these models causing an increase in the time required for the development of new effective therapies ⁷³. For all these reasons, this approach has been substituted by 3D culture. Indeed, since the importance of *in vitro* systems for cancer studies, the number of publications about the development of 3D models have been increased in the last 10 years (Fig.1.10). Importantly, also the design of lung models has been expanded over the years, suggesting an increasing interest in the analysis of this organ and its associated diseases (e.g. cancers).

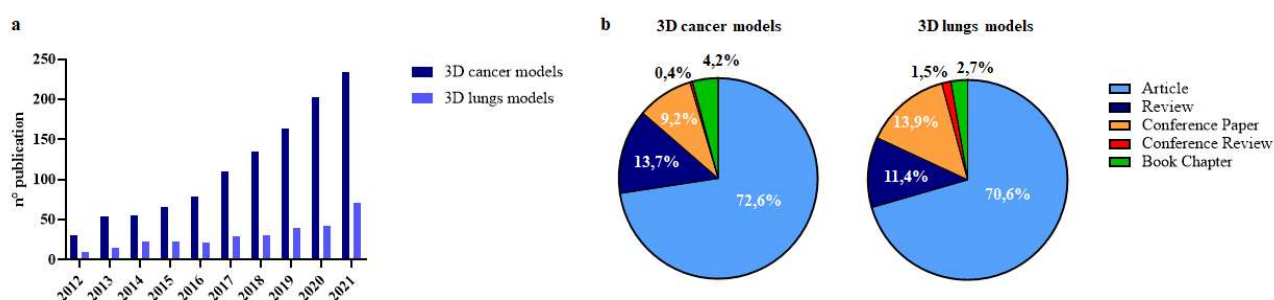


Fig. 1.10: Quantity (a) and types (b) of published works from 2012 to 2021 listed on Scopus about 3D cancer/lungs models.

1.4.3 3D culture systems: Spheroids

Several 3D systems have been developed over the years. Among those, one of the most used are spheroids, which consisted of cells organized in a spherical shape with a diameter of 200 μm or more. Inside this structure, three different concentric zones can be identified (Fig. 1.11a):

- an external one in which cells are highly proliferating and migrating,
- a middle part where the cells are quiescent because of the lower amount of nutrients,
- a necrotic zone in which oxygen and nourishment are not sufficient to maintain a high level of cell viability.

Overall, spheroids culture system is a promising method to improve cell viability and proliferation, to facilitate cell-cell interactions and to maintain the level of released signaling molecules close to the one founds *in vivo* ⁷⁴.

Usually, they are obtained by scaffold-free techniques, particularly, the most common methods are : (I) liquid overlay, which is based on the interruption of cell adhesion by using special non-adhesive substrates, and (II) hanging drop, which takes advantage of surface tension and gravitational forces (Fig. 1.11b) ⁷⁵. Importantly, the steps required for spheroids formation must be fully controlled to obtain a structure with standard properties. Multiple parameters, such as mean diameter, size heterogeneity, number of spheroids, and duration of cultivation, must be optimized. For instance, it has been shown that the modulation of the number of initial cells and the time required for spheroids formation influenced drug response, because of changes that occurred in surface topology and local stiffness ⁷⁶. Spheroids have been also employed for the studying of NSCLCs, specifically, insightful features on cell-cell adhesion, chemoresistance, tumor heterogeneity and extracellular matrix deposition have been analyzed. Indeed, multicellular aggregates allow the evaluation of the cross-talk between the tumor cells and the stroma revealing interactions which have been completely lost in traditional 2D culture ⁷⁷.

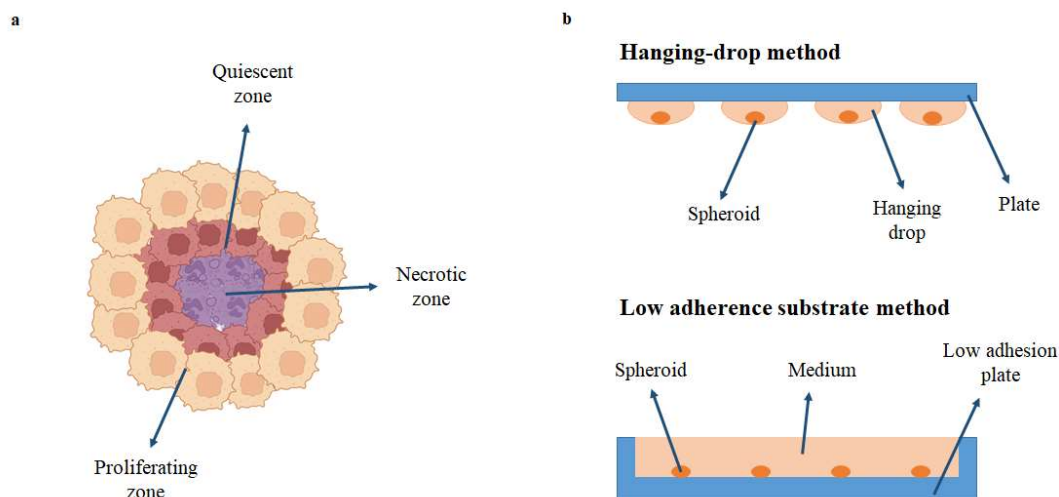


Fig. 1.11: Schematic representation of spheroid structure (a) and most common preparation methods (b).

1.4.4 3D culture systems: Organoids

Another widely used strategy to develop 3D models is the growth of organoids, which are self-organizing 3D cultures, mimicking human organs, derived from pluripotent stem cells (PSCs) or tissue stem cells (TSCs). Organoids are helpful in biological and biomedical research, because of their similarity to physiological conditions. Moreover, they can be used when animal models fail in reproducing mechanisms that occur in human development or disease. Importantly, organoids can also be derived from biopsies of tissues of interest ⁷⁸ and several culture environments have been optimized according to the type of organoid required. For instance, natural or synthetic compounds act as supportive matrices for intestinal and gastric organoids, whereas cerebellar and optic cup structures prefer suspension culture systems supplemented or not with ECM proteins. Interestingly, air-liquid interface culture can be used for the development of kidney organoids ⁷⁹. Obviously, the designing of disease models, including cancer, became easier thanks to the possibility of using multicellular structures. Interestingly, tumor organoids can be derived from several sources: primary tumors, metastatic lesions, circulating cells and cells from liquid effusions. Further, they could be formed starting from single cells obtained from the digestion of the source or from tumor fragments according to the analysis required ⁸⁰.

Although the structural complexity of this organ, protocols for deriving lung organoids have been optimized (Fig. 1.12). Starting from hPSCs, differentiation into foregut spheroids is obtained in one week. Subsequently, those spheroids are embedded into Matrigel and cultured in a medium supplemented with high levels of Fibroblast Growth Factor 10 (FGF10) and 1% of fetal bovine serum (FBS) to obtain human lungs organoids that consist of the airway-like structure surrounded by mesenchymal cells. These models allow the studying of the interactions that occur between mesenchymal and epithelial cells during organ development. On the contrary, if foregut spheroids are grown in a serum-free medium containing FGF7, an inhibitor of a serine/threonine protein kinase called glycogen synthase kinase 3 (CHIR-99021) and an all-trans retinoic acid (ATRA), branch-like organoids which expressed proximal and distal airway markers are derived. Importantly, they can give rise both to airway-like and to alveolar-like cells ⁸¹. Since its high diffusion and mortality rate, lung cancer organoids show several advantages in the studying of molecular features, such as gene transcription and protein expression, tumorigenesis mechanism, microenvironment properties and their interactions with tumor cells, high through-output drug screening and the establishment of personalized therapeutic approaches ⁸². For instance, Kim and colleagues successfully obtained organoids from different subtypes of lung cancers: adenocarcinoma, squamous cells and adenosquamous carcinoma, large cell cancer and SCLCs. Importantly, obtained models fully resemble the histological properties and the marker's expression of the parental tumors ⁸³.

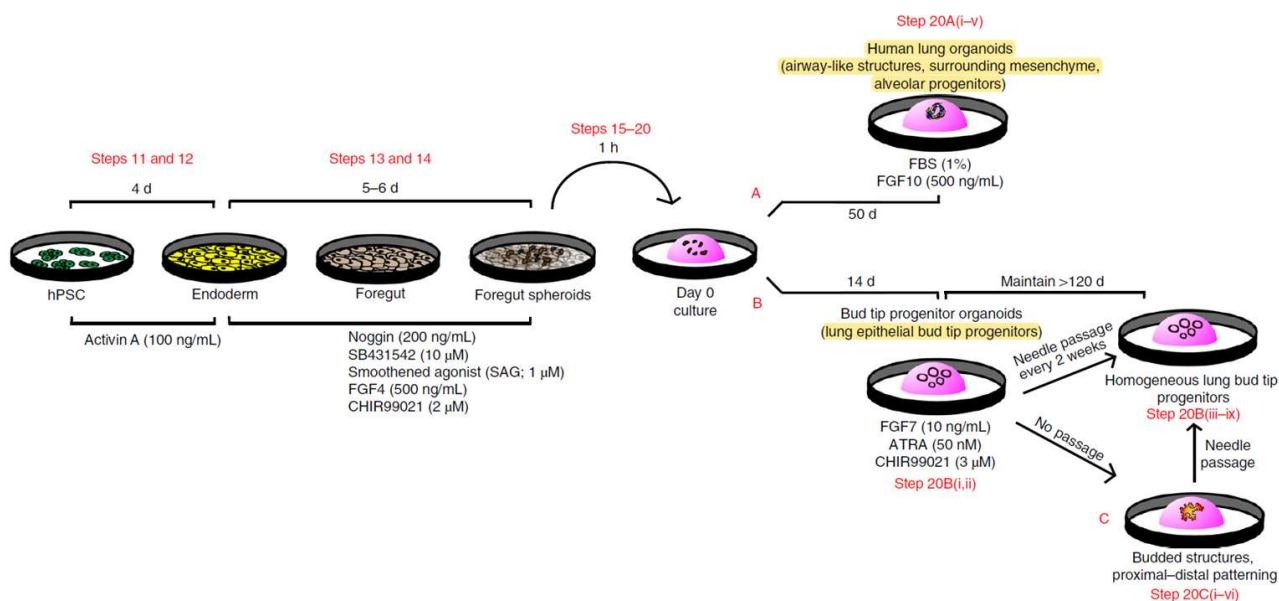


Fig. 1.12: Schematic protocol for obtaining lung organoids. Adapted with permission from Miller et al., 2019⁸¹.

1.5 Matrices for lung 3D *in vitro* culture models

Since the importance of the development and optimization of *in vitro* models to reduce animal use in research, the study of biocompatible matrices for the establishment of 3D culture systems becomes crucial. In this section, the main natural and synthetic compounds and their use will be analyzed.

1.5.1 ECM-derived matrices

Extracts of basement membrane have been widely used in biological research for the establishment of 3D culture. The best known and analyzed are BME (i.e. basement membrane extract) and Matrigel (i.e. extracted from Englebreth-Holm-Swarm tumors in mice), which is similar to a conserved, early developmental basement membrane. In the ECM matrix-derived the most abundant components are laminin-111, collagen IV, entactin, and heparan sulfate proteoglycan, growth factor and proteases. Thanks to that composition, a plethora of applications can be identified. The most common *in vitro* analysis where these matrices are used consists of endothelial cell tube assay, 3D culture of tumor cells (i.e. multicellular spheroids and co-culture, organoids) and invasion assay. Further, because of its biocompatibility, basement membrane extract can also be used in *in vivo* experiments, such as PDX⁸⁴⁻⁸⁶. Lately, well-defined protocols for deriving adenocarcinoma organoids from patients' biopsies in Matrigel or BME have been optimized to reach a high success rate (e.g. higher than 80%). These organoids facilitate the histological analysis of the specimens and further molecular analyses, which guided the clinicians in selecting the right anti-tumoral therapies⁸⁷. On the other hand, the ECM matrix-derived shows some drawbacks.

Unfortunately, basement membrane extracts show high batch-to-batch heterogeneity in terms of composition and mechanical properties. Another drawback is the need for temperature-controlled environment to avoid early polymerization, this issue is quite difficult to reach and maintain for *in vivo* applications. These limitations lead to inconsistency in obtained results, and the research of synthetic alternatives has acquired huge importance in recent years ⁸⁸.

Another widely used matrix for 3D culture systems is Collagen Type I. Indeed, it is an actual component of the ECM, hence, it shows high biocompatibility, thus, it has been successfully used to establish *in vitro* cancer models (e.g. breast ⁸⁹ and lung ⁹⁰) or to analyze cells' behavior in a 3D environment ⁹¹. Its main limitations are the poor mechanical properties and the faster degradation rate during long-term cell culture. Thus, chemical modifications are usually required to improve collagen-based hydrogels stability ⁹².

Another strategy to obtain ECM matrix-derived is to use Hyaluronic Acid (HA), which is a glycosaminoglycan present in the extracellular environment. Especially, it is the main component of the ECM in neuronal tissue, hence, it could be used as a scaffold for the development of brain organoids. HA shows several advantages, including biodegradability, biocompatibility, and the presence of functional groups, which make it suitable for 3D culture and tissue engineering. Despite that, its important limitations are the short residence time and lack of mechanical integrity, which could be overcome by chemical modifications and stable crosslinking ⁹³.

1.5.2 Natural and synthetic alternatives to ECM

As described in the previous paragraphs, ECM-derived matrices show great advantages, as biocompatibility and mimicking of the physiological environment. Unfortunately, the costs, the batch-to-batch variability and the poor mechanical properties limited their use and required chemical modifications to improve their features. To overcome these limitations, the selection and optimization of other polymers have been necessary.

One of the first candidates to substitute ECM -derived matrix is Alginate. It is a linear anionic and water-soluble polysaccharide polymer composed of β -(1-4)-D-mannuronic (M-blocks) and α -L-guluronic acid (G-blocks). Importantly, the ratio of the two components and structure strongly influence several properties of the final hydrogel, whereas the viscosity depends on the solution pH. It can be derived from different kinds of seaweeds and bacteria. The polymerization of the hydrogels could be obtained with different methods, among which ionic or covalent crosslinking, phase transition caused by temperature modulation, cell crosslinking, free-radical polymerization and click-chemistry ⁹⁴. Alginate presents several advantages: for instance, it is abundant in nature and it derives from renewable sources, hence, it is quite inexpensive; further, it is biocompatible, non-toxic and its properties can be easily modulated by changing the amount of the constituent molecules. Thus, it has been used for drug and cell delivery, as a substrate for cell culture and as a scaffold for tissue engineering ⁹⁵. One of its main drawbacks is the lack of stability over time, due to the ionic exchange with the surrounding medium which leads to gel dissolution. Thus, usually, alginate is chemically modified to allow covalent crosslinking and avoid

premature hydrogel degradation. Further, since alginate is obtained from natural sources, a purification process is required to remove many impurities, like heavy metals, endotoxins and proteins that could limit the use of this material for biomedical applications ⁹⁶. Moreover, inter-grade and inter-batch variability were detected ⁹⁷.

A valid alternative to Alginate is methacrylated gelatin or GelMA. It can be synthesized through the direct reaction between gelatin and methacrylic anhydride, during which the reactive amine and hydroxyl groups on gelatin were substituted by the methacryloyl groups. The polymerization of the material is achieved through the crosslinking induced by radical reactions induced by a photo-sensible molecule, called photoinitiator. Interestingly, the features of the final hydrogels can be easily modulated. A modification in the degree of substitution of the gelatin during the synthesis results in a variation of the mechanical properties of the polymerized matrix. Moreover, the elastic modulus value depends on different parameters as temperature and polymer concentration ⁹⁸. Further, since it is derived from gelatin, GelMA promotes cell adhesion, migration and proliferation ⁹⁹. Gelatin is hydrolyzed collagen type I, which in turn is the major component of several tumor microenvironments, thus, GelMA has been successfully used for the study of cancer models (e.g. breast carcinoma ¹⁰⁰, bone cancer ¹⁰¹, colorectal cancer ¹⁰²). The porous structure of the hydrogel allows the formation of spheroids, which can be tested through invasion assay or in vivo metastatic potential. Moreover, those models could be used for the analysis of drug response and high-throughput drug screening ¹⁰³. GelMA-based hydrogels have been successfully used for modeling several tissues and organs: neuronal and cardiovascular systems, liver, bone, skeletal muscle and cartilage, spinal cord, lung and skin ¹⁰⁴.

1.6 Microfluidic device for biomedical applications

Microfluidic devices represent a powerful tool in biomedical analysis, diagnostic and in high-throughput processing (e.g., drug screening), because they allow the automation of those processes, which usually require long and complex preparation. Further, microfluidic devices played an important role in the early diagnosis of human disease and the selection of suitable therapeutic protocols. In this paragraph, the main applications and techniques will be described to underline the importance of these devices in the biomedical field and, in particular, in cancer research.

1.6.1 Lab-on-a-Chip

For many years, researchers focused their attention on the designing of microfluidic platforms to obtain Lab-on-a-Chip (LoC) devices. They consist of integrated circuits of a few millimeters, able to perform several processes, which were usually conducted in wet laboratories. Their main advantages are the efficiency, the low amount of reagents required for the reactions, the quicker analysis, the automation and the cheapness. In the years, several techniques have been developed to obtain LoC including photolithography, soft lithography, laser micromachining, screen printing, nanoimprinting and 3D printing. Importantly, the detectors included in the

devices can be electrical, electrochemical, mechanical or optical transducers ¹⁰⁵. In cancer research, one of the main applications of LoC is in liquid biopsies (Fig. 1.13). Indeed, microfluidic-based digital PCR could be used for the successful recognition of circulating tumor DNA whose concentration is usually low. Instead, other chips are optimized for the identification, the isolation and the analysis of circulating tumor cells (CTCs) ¹⁰⁶. For instance, Gwak and colleagues developed a microfluidic device able to recognize and separate two types of extracellular vesicles associated with breast cancer ¹⁰⁷. For lung cancer diagnosis, several microfluidic devices have been or will be included in clinical trials to validate their capacity to detect CTCs and allow early diagnosis, which in turn could ameliorate patients' life expectancy after diagnosis ¹⁰⁸.

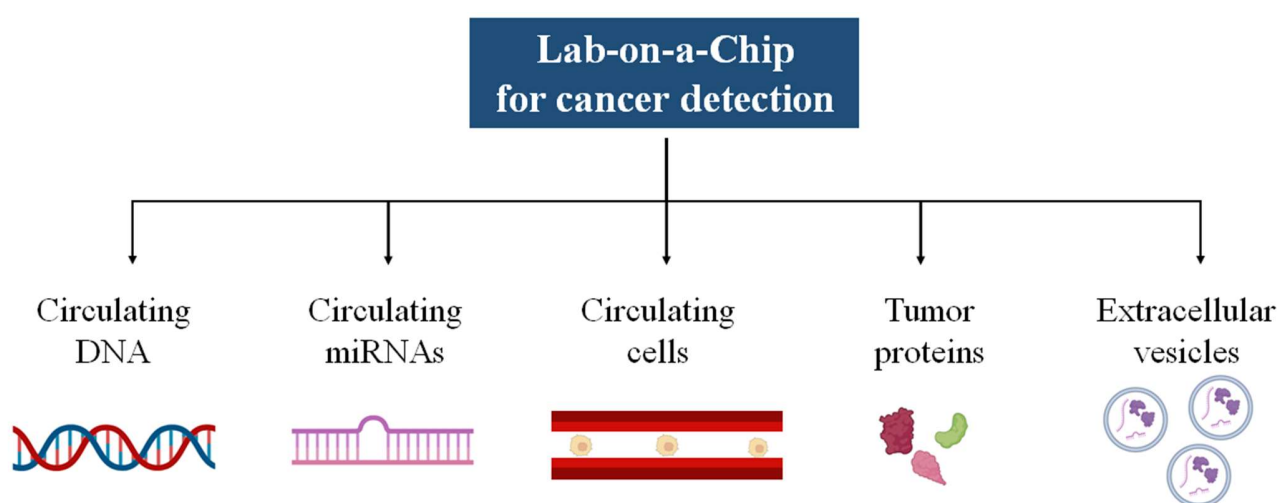


Fig. 1.13: Schematic representation of the main uses of LoC in cancer diagnosis. Created by BioRender.

1.6.2 Organs-on-chip

The designing of LoCs for biomedical applications leads the studies towards microfluidic chambers able to host culture of miniaturized human tissue. Indeed, an Organ-on-Chip (OoC) is a microfluidic device that contains continuously perfused chambers inhabited by living cells arranged to simulate tissue- and organ-level physiology ¹⁰⁹. Indeed, during the last decades, the designing of OoCs has acquired huge importance.

Specifically, the main fields of application are regenerative medicine, disease modeling and personalized medicine. Moreover, the complexity of these devices strongly increased over the years ¹¹⁰. They consist of miniaturized organs which are recreated into chip-like arrays. The advantages of these devices are the possibility to manipulate or modulate the microenvironment in which cells reside, the establishment of 3D culture and the capability to connect different organs to recapitulate the cross-talk that happens in *in vivo* models. Importantly, several models have already been developed, such as heart, intestine, brain, bone, liver, adipose tissue and lung¹¹¹. Although its complexity, lung tissue has been modeled in different types of OoCs to mimic and analyzed

inflammation, drug responses and mechanical stimuli ^{112,113}. For instance, microfabricated microfluidic system containing a flexible membrane coated with ECM molecules (i.e. fibronectin or collagen) could be used to grow alveolar and endothelial cells creating a liquid-air interface to mimic the alveolar compartment. Moreover, cyclic stretching produced by a vacuum pump, resembles the physiological movements linked to breathing, while the flow of medium inside the microfluidics mimics the fluid shear stress applied on the endothelial cells ¹¹⁴. Similarly, Baptista and colleagues designed a biomimetic membrane, which recreated the spherical appearance of the microenvironment where the alveolar epithelial cells usually reside (Fig. 1.14) ¹¹⁵. Microfluidic devices could also be used to assess changes that occur in cell behavior during cancer progression and metastasis formation, to detect mutations and evaluate efficiency of drugs and therapeutic protocols ¹¹⁶⁻¹¹⁸. These devices show several advantages, among which controlled microenvironment, the presence of a hemodynamic-like compartment and physiological biomechanical and biochemical stimuli. Unfortunately, the absence of optimized and standardize protocols and difficulties to scale-up represent important limitation. In the years, model of diseased lungs have been developed: i.e. COPD, asthma, lung cancer, fibrosis and pulmonary thrombosis ¹¹⁹.

Beyond OoCs, microfluidic devices allow the establishment of perfused 3D culture in which continuous fluid flow provides nutrients supply and waste removal, as well as, the creation of chemical gradients that are usually present in *in vivo* tissue ¹²⁰. For instance, it has been shown that perfused 3D culture provides a reliable platform for stem cell culture and testing of drug effects ¹²¹. Importantly, microfluidic arrays that supports cell culture can be implied in long-term high-throughput assay (i.e. drug screening, bioinformatics ad quantitative cell biology)¹²². Further, the laminar flow that is strictly controlled in microfluidic systems, simulate diffusion gradients of molecules that guide the differentiation, proliferation, and migration of various cell types *in vivo*. In addition, through specific geometric and structural designs, the continuous flow can generate fluidic shear stress or interstitial fluid flow to promote the establishment of specific microenvironments ¹²³. For all that reason, the development of an OoC based on 3D perfused cell culture may represent a crucial step forward the designing of *in vitro* devices able to study human diseases and to predict the *in vivo* response of drug treatments.

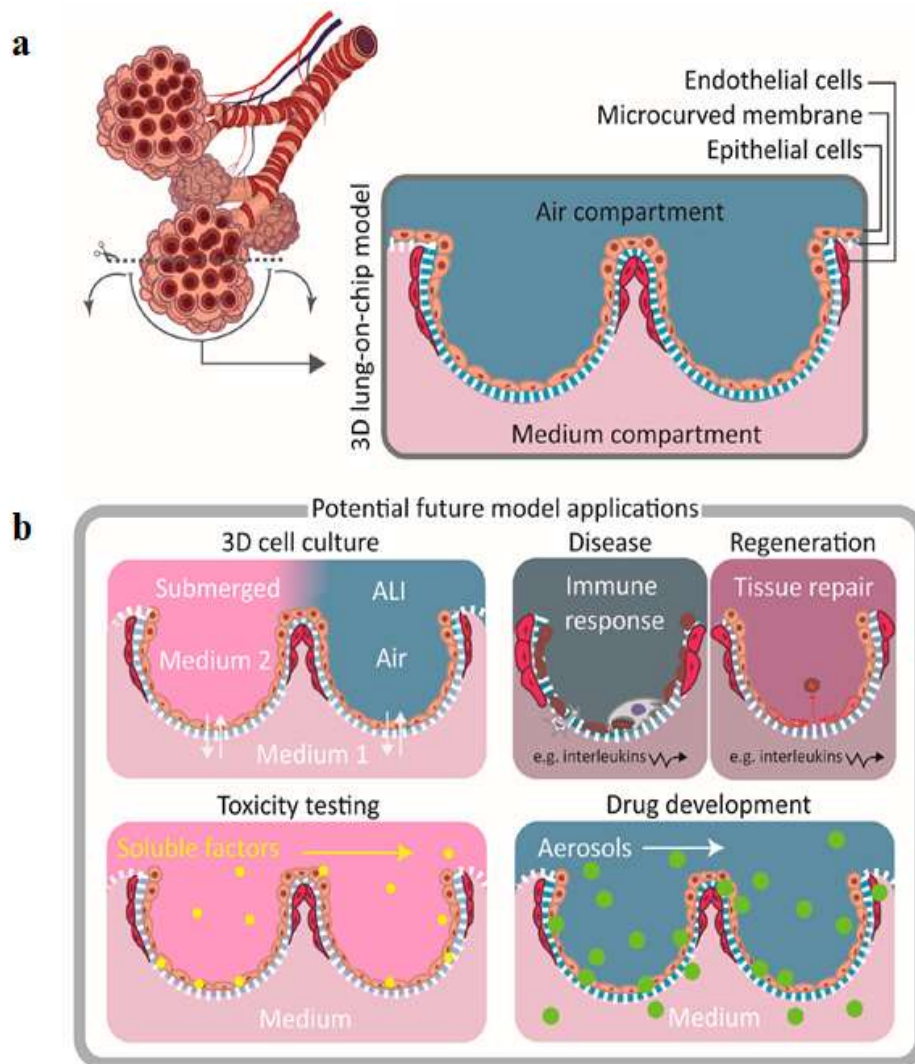


Fig. 1.14: Concept of a 3D lung-on-chip model (a) and applications (b). Adapted with permission from Baptista et al., 2022 ¹¹⁵.

1.7 3D printing technologies for biomedical application

3D printing is an additive manufacturing technology that lately has been applied in biomedical research¹²⁴. For instance, stereolithography, which will be described below, has been used for the easier production of LoCs for medical applications¹²⁵.

Merging additive manufacturing and biological research needs is possible to realize 3D printed scaffolds at high resolution to fully mimic the structure of human organs and partially substitute animal models; a new trend, called 3D bioprinting, has been rising. Bioprinting can be defined as a computer-aided transfer process for patterning and assembling living and nonliving materials¹²⁶. Several approaches have been optimized during the last decades. In this paragraph, a general overview on the main techniques used and their applications will be shown (Fig. 1.15).

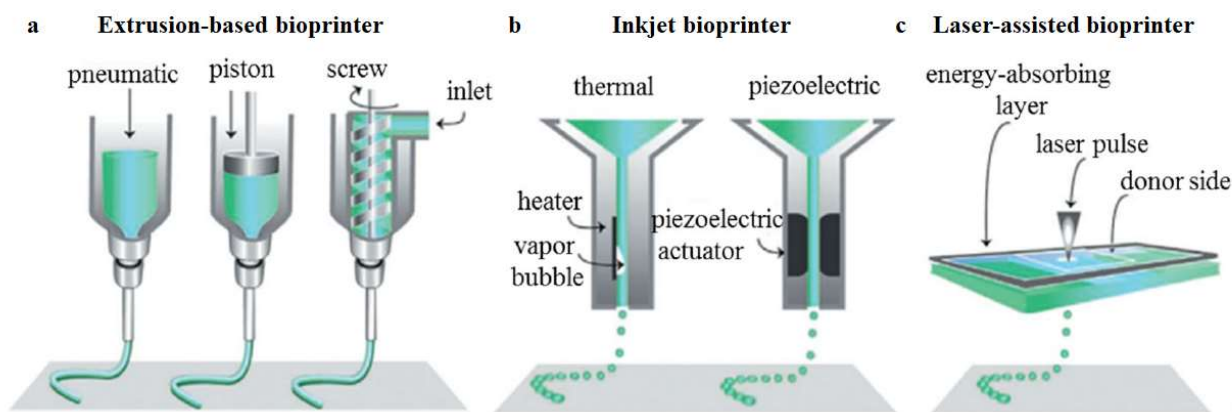


Fig. 1.15: Schematic representation of the most used techniques for 3D printing for biomedical application: extrusion-based (a), inkjet-based (b), and laser-assisted (c) printing. Adapted with permission from Das and Basu, 2019¹⁴⁵.

1.7.1 Inkjet-based bioprinting

The inkjet-based 3D printing is a high-resolution (e.g., output up to 50 μm), high-speed and unexpensive process used for biofabrication of 3D structures. An important advantage is its compatibility with many biological materials. The solutions are dispensed thanks to thermal, piezoelectric or microvalve impulse. In the first case, rapid electrical heating of the printing head promotes droplet deposition, whereas, in the piezoelectric configuration an acoustic stimulus induces regular droplet release^{127,128}. Before printing, several parameters are investigated and optimized: ink's density and viscosity, surface tension, printing velocity, droplet length and nozzle diameter¹²⁹. Although the advantages, this technique presents several drawbacks that reduce its use in 3D bioprinting. For instance, shear stress and high temperature have an impact on cell viability. Moreover, to avoid clogging formation, which may block droplets formation, cell density is quite low compared to the physiological

conditions. Since the printing resolution is determined by the interaction between adjacent drops, the surface tension and wettability can have a negative impact on the fidelity of the final scaffold. Further, the shape and size are strictly regulated by liquid-to-solid phase transformation ¹³⁰.

This technique has been successfully used for models of cartilage, bone (e.g. deposition of cell-loaded fibrin-collagen hydrogel), neural (e.g. deposition of cells suspended into PBS), cardiac and vascular tissue and skin ¹³¹. Inkjet-based has been used also for the *in vitro* modeling of lung alveolar structure (Fig. 1.16). Indeed, Kang and colleagues successfully printed three different functional layers to mimic the alveolar epithelium, endothelium and the contribution of the basement membrane. Specifically, endothelial cells and the epithelial ones (i.e. AT1 and AT2 cell lines) were dispersed into cell culture medium, whereas collagen was loaded with fibroblasts to mimic the ECM contribution. Importantly, the high resolution obtained and the complexity of the structure closely resembles the one found *in vivo* ¹³².

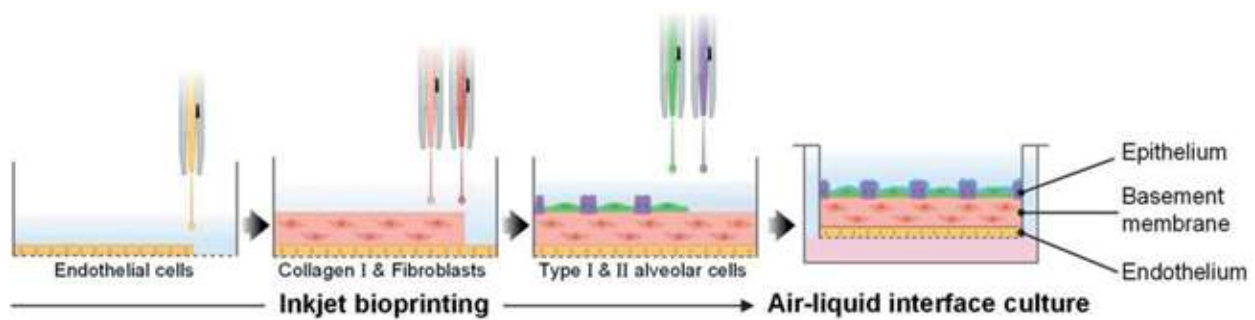


Fig. 1.16: Schematic protocol of inkjet process for the printing of a 3D alveolar barrier. Adapted with permission from Kang et al., 2021 ¹³².

1.7.2 Extrusion-based bioprinting

Extrusion-based printing is the most used technology of 3D printing in biomedical applications. The possibility to customize printers with multiple heads allows to obtain 3D printed scaffolds with regional differences in terms of materials, cell types and density, and in signaling molecules' amounts. In bioprinting, the two main drawbacks are the cell exposure to shear stress during the flow and the definition of the printability of selected bioinks, due to the lack of standardized methods ¹³³.

The ink dispensing could be mediated by three different methods, specifically (I) pneumatic-based, (II) piston-based and (III) screw-driven systems. (I) In the pneumatic one, a controlled air pressure is applied to induce ink flow and produce a scaffold with relative simplicity. (II) The piston-driven approach allows a higher control over the flow, whereas, (III) the screw-driven is preferable for very viscous materials. Importantly, the printing

procedure decreases cell survival because of the shear stress to which cells are exposed during the process. Interestingly, the pneumatic-based bioprinting seems to preserve better viability than the other two methods¹³⁴.

Moreover, this approach can be used to obtain 3D printed cancer models that resemble *in vivo* structures and features. For instance, Wang and colleagues showed that lung cancer cells could be printed without any decrease in viability and proliferation rate. They demonstrated again that the invasive behavior and the gene expression are changed compared to the traditional 2D culture¹³⁵. A more complex model has been obtained by combining 3D printing and external stimuli that mimicked the breathing, the diffusion of gas and nutrients and the blood flow (Fig. 1.17). The aim is the designing of *in vitro* lung normal tissue or cancer, to better predict the drug response and metastatic capability acquisition^{136,137}

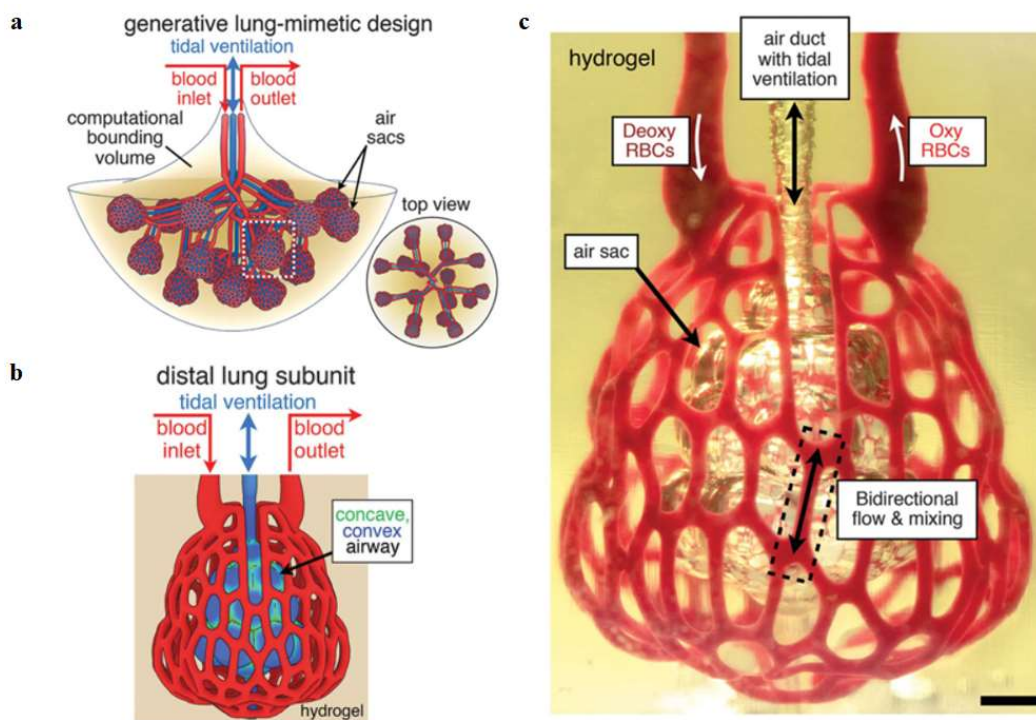


Fig. 1.17: Schematic representation of the steps for the designing of a 3D printed lung-mimetic device. a) Lung mimetic design with an inlet and outlet vascular network and branched population of distal alveoli. b) Schematic representation of alveolar unit. c) Picture of a printed alveolar unit. Adapted with permission from Grigoryan et al., 2019¹³⁷.

One of the challenges of this approach is the possibility to create a microfluidic structure inside the printed scaffold to mimic the physiological vasculature. A widely used solution is the use of sacrificial materials which can be easily removed after the printing and the polymerization of the cell-laden hydrogel¹³⁸. The main used temporary inks are Pluronic F127¹³⁹, Carbopol¹⁴⁰, Agarose¹⁴¹, Gelatin¹⁴² or Alginate¹⁴³. Interestingly, all of

those can be removed by decreasing (e.g. Pluronic F127) or increasing (e.g. gelatin) the temperature, by physical extraction (e.g. Agarose), by washing (e.g. Carbopol) or by using chelating agents (e.g. Alginate) ¹⁴⁴.

1.7.3 Laser-assisted bioprinting

Laser-assisted printing is the only approach that does not require nozzle presence. Indeed, it is a free dispensing process in which inks' viscosity and/or cell concentration do not negatively affect printing efficiency and resolution. The printhead setup comprises a laser transparent material, a laser-absorbing media to avoid cell damage induced by high-power laser, and the bioinks (e.g. cell-loaded hydrogels). The laser impulse creates on the bioink film a high local pressure. The pressure of absorbing media generates bubbles toward the bioink film. The expansion and collapse of the bubble create a jet, which leads to the release of the droplets which will be transferred to the printing substrate ^{145,146}. This technique has several advantages: (I) it does not create mechanical stresses resulting in high cell viability (>95%), then (II) it can be used with the material in the solid or liquid phase and (III) the resolution obtained is near to 10 μm . Although that, the main drawbacks are its expensiveness, the low availability of bioinks, the thermal damage due to laser irradiation and the tricky preparation ¹⁴⁷. For instance, thanks to this technology, Michael and colleagues successfully recreate a multi-layered skin equivalent, which can be transplanted *in-vivo* with the aim of treating burned patients ¹⁴⁸.

1.7.4 Stereolithography-based printing

As mentioned earlier, 3D printing could be implied also in the production of microfluidic devices, like LoCs and OoCs. Indeed, it represents a huge innovation in terms of resolution, easy handling and versatility compared to the classical soft lithography. Importantly, the main used strategies are based on photocurable resins, such as stereo lithography appearance (SLA), digital light processing (DLP), liquid crystal display (LCD), multi-jet printing (MJP), two-photon 3D printing (TPP), holographic technologies ¹⁴⁹.

Specifically, SLA setup is based on a single computer-controlled laser beam that builds structure in a bottom-up manner (Fig.1.18a). Indeed, only a thin layer of resin is illuminated and cured. This technique presents some limitations, among which are the low speed rate and the resolution. Further, few resins are available to be used in this approach ^{149,150}.

However, to improve the velocity of the conventional SLA process, DLP-based printing has been developed. The main difference between these two approaches is the top-down process (Fig. 1.18b). Then, a digital mirror device (DMD), which is an array of micro mirrors, modulates the laser beam to irradiate an entire layer, which in turn can be polymerized at the same time ¹⁵¹. For this reason, nowadays, DLP-based procedure is the most used one.

Its applications include the designing of medical devices, tissue engineering and products for drug discovery, delivery and development ¹⁵². Importantly, resins suitable for DLP-based printing have to fulfill several

parameters. For instance, it is necessary to keep low the viscosity of the formulations, indeed, for the printing of a new layer the liquid should flow into the gap between the bottom surface and the previously cured layer. If this does not happen, the printing procedure will fail ¹⁵³. Further, used formulations have to be transparent in the visible light and UV spectra to allow the curing during the process and biological analysis. Indeed, DLP-printed samples could be used as OoCs and the transparency is required form many assays which required fluorescence signal measurement ¹⁵⁴.

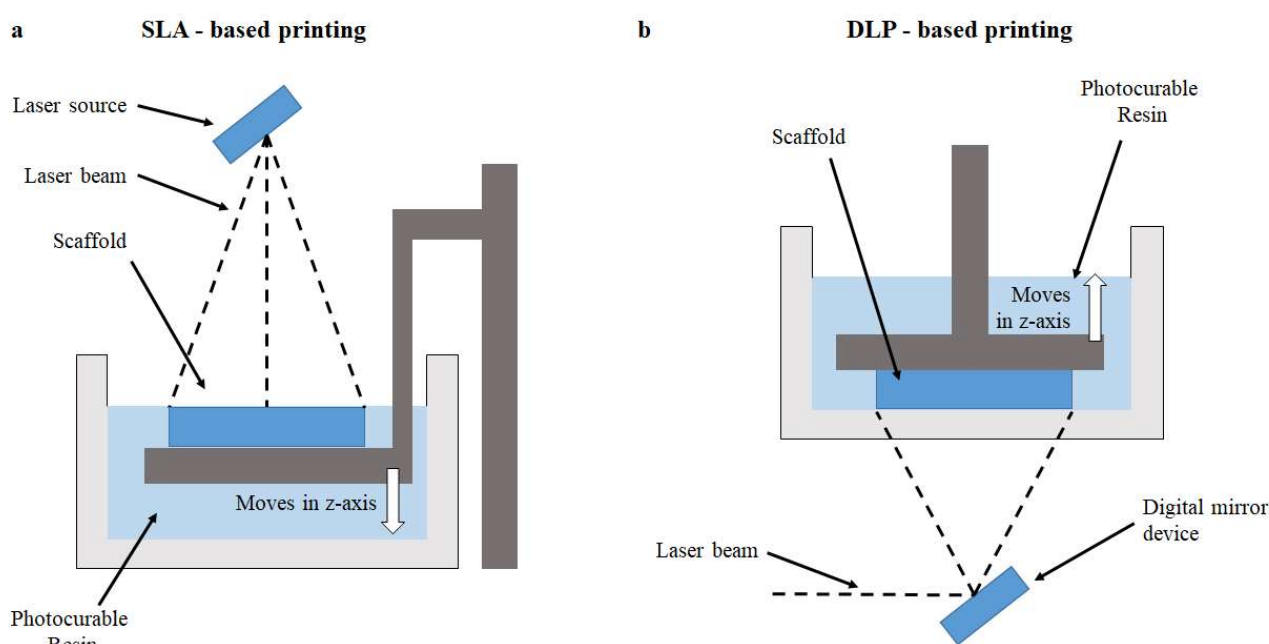


Fig. 1.18: Schematic representation of SLA (a) and DLP (b) –based printing.

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Chapter 2

Development of GelMA-based models of lung adenocarcinoma

Abstract

This chapter describes the synthesis and characterization of a hydrogel formulation based on gelatin methacrylate (GelMA). The aim is the development of a scaffold that supports the growth of a tumor model, in particular the Non-Small Cell Lung Cancer (NSCLC) niche. Specifically, GelMA synthesis was modulated to obtain different degrees of functionalization, i.e. the introduction of different contents of reactive methacrylic groups on gelatin backbone, by chemical functionalization. These materials were loaded with cells and then photocured to obtain the desired scaffold. Importantly, *in vitro* cell analysis on GelMA matrix showed that changes in hydrogel properties and concentration had an impact on the cell proliferation rate, of both adenocarcinoma cell lines (A549 and H1299) and normal lung fibroblasts (MRC-5). Moreover, further cellular and molecular analysis, demonstrated the capability of selected GelMA hydrogels to influence cell behavior and the feasibility of using them as matrix for the establishment of long-term cell culture.

2.1. Introduction

NSCLC is a widespread tumor classified in heterogeneous subtypes according to the cell of origin and molecular features^{1,2}. Disease progression is linked to a complex microenvironment, which includes several cell types and matrix components. Indeed, it is widely accepted that cancer associated fibroblasts (CAFs), immune and endothelial cells strongly influence cancer growth, invasion and progression³. Moreover, pervasive angiogenesis induced by tumor cells carries the nutrients and signaling required for cancer survival⁴. On the other hand, it is highly challenging to fabricate an *in vitro* model of NSCLS, reproducing the aforementioned high heterogeneity and the microenvironment role¹. Certainly, traditional 2D culture fails in this, whereas 3D environment (e.g. scaffolds) could better sustain cell culture, with stimuli alike the physiological ones⁵. For 3D models, several matrices have been tested, as Matrigel⁶, hyaluronic acid⁷, alginate⁸, collagen⁹ and gelatin methacrylate¹⁰. In the present study, the focus is gelatin and its modification. Gelatin is a polypeptide derived by partial hydrolysis from collagen, which is commonly present in the connective tissue of several mammals and fish. Specifically, it is derived from Collagen type I through the unfolding of the triple helical conformation in random-coiled domains and depending on the source, gelatin could have different physical properties. For instance, fish-derived gelatin shows a lower melting point and higher viscosity compared to the ones derived from bovine and pigs¹¹. Importantly, gelatin presents arginine-glycine-aspartic acid (RGD) residues which allow integrin-mediated cell adhesion¹². Nevertheless, the use of gelatin as hydrogel is limited by its low melting point, around 28°C, and, the poor mechanical properties. In this context, its methacrylation and consequent cross-linking can be a suitable

strategy to overcome these drawbacks. Indeed, the cross-linked GelMA does not melt at 37°C, which is the classic cell culture temperature, and the mechanical properties are improved in a high temperature range (from 15°C to 45°C). Cross-linking can be mediated by the introduction of a photoinitiator, that starts the reaction of polymerization under UV-visible light irradiation by promoting the formation of radical residues of unsaturated methacrylic groups ¹³. Importantly, gelatin modification is not cell toxic, and the so-obtained hydrogel preserves enough adhesive site to promote cell attachment ¹⁴. Furthermore, the degree of functionalization (DoF) of gelatin influences the mechanical properties, due to different cross-linking density, and consequently cell behavior ¹⁵. Thus, the versatility of GelMA chemical, physical and mechanical properties make it suitable for the establishment of 3D printed organ-on-chip models. The use of GelMA in bioprinting has been already investigated in several works, showing also the possibility to produce 3D printed vascularized scaffolds ^{10,16}. Moreover, GelMA supports multi-cellular culture and it was used to mimic hepatic fibrosis ¹⁷ or skin tissue ¹⁸ by adding to the matrix, the different cell types that are found in physiological conditions.

This chapter deals with GelMA formulations optimization to obtain hydrogels able to support cell growth and viability. Specifically, two immortalized cell lines of lung adenocarcinoma, A549 or H1299, were used. Moreover, to study the effect of the stroma, normal lung fibroblasts, MRC-5, were added to both models. Several *in vitro* cell analyses were carried out to assess cell proliferation and viability, in mono-culture as well as long-term co-cultures. Further, fibroblasts maintenance was evaluated by cytofluorimetric analysis. Once the optimized GelMA formulations were selected, preliminary molecular studies were performed to uncover the role of fibroblasts in our matrices. Specifically, the expression of ECM related genes and proteins was analyzed.

2.2. Materials and Methods

2.2.1. Materials

For GelMA synthesis, type B gelatin from bovine skin (G6650), Dulbecco's Phosphate Buffered Saline or DPBS (D1408), Methacrylic anhydride or MA (276685) and Cellulose Membrane were obtained from Sigma Aldrich (Milan, Italy). The photoinitiator, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate or LAP (900889), was purchased from the same company. Tumor cell lines A549 and H1299 were kindly provided by Valentina Monica from University of Turin and normal lung fibroblasts, MRC-5 (CCL-171), were obtained from ATCC. Benchstable RPMI 1640 medium with GlutaMax (A4192302) and BenchStable MEM medium with GlutaMax (A4192202) were purchased from ThermoFisher, whereas the supplements fetal bovine serum or FBS (F9665), penicillin/streptomycin, sodium pyruvate (S8636) and MEM non-essential amino acids solution 100X (M7145) were bought from Sigma-Aldrich. For viability assays, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (M2003) and Live/Dead staining kit (04511-1KT-F) were taken from Sigma-Aldrich. Moreover, Collagenase from Clostridium (C9407), Triton X-100, 10X casein and Bovine Serum Albumin (BSA) were obtained from the same company. Paraformaldehyde 4% in PBS (J61899.AK), PierceTM BCA Protein

Assay Kit (Cod. 23225) were bought from ThermoFisher Scientific. Anti-fibronectin (ab2413), anti-collagen type 1 (ab260043) and anti-alpha-smooth muscle actin or α -sma (ab7817) antibodies were purchased from Abcam. Goat anti-Mouse IgG Alexa Fluor 546 (A-11003) and Goat anti-Rabbit IgG Alexa fluor 488 (A-11008) were taken from Invitrogen. Reagents required for real time analysis, UltraPure DNase/RNase free distilled water (10977035), PowerTrack™ SYBR Green Master Mix (A46111), High capacity cDNA reverse transcription (4368814), were bought from ThermoFisher Scientific and primers were provided by Kaneka Eurogentec S.S. (Belgium).

2.2.2. GelMA synthesis

GelMA was synthesized following a protocol previously described¹³. In particular, to prepare 10 w/v% GelMA, 10 g of type B gelatin from bovine skin was dissolved into 100 mL DPBS at 50°C for 1 hour. Then, MA was slowly added to the solution and the reaction lasted 2 hours in the dark at 40°C, under continuous stirring. The amount of added MA was modulated as described in Tab. 2.1. The reaction was stopped by adding 100 mL of DPBS at 40°C to avoid precipitates formation and the solution was dialyzed against dd-H₂O with cellulose membrane (molecular weight cut-off 14kDa) for 1 week at 40°C. At the end, GelMA was freeze-dried for 1 week and maintained in the dark at RT until use.

GelMA Type	MA (mL/ 10 g Gelatin)
Low	2
Medium	4
High	8
Very High	20

Tab. 2.1: MA quantity added for each GelMA type to reach different degrees of substitution.

2.2.3. Evaluation of GelMA degree of methacrylation

For the assessment of gelatin's degree of functionalization (DoF), different types of GelMA were dissolved in DPBS at a concentration of 2 mg/mL. Then, 300 μ L of each solution was mixed with 600 μ L of fluorolaldehyde o-phthalaldehyde reagent solution. Gelatin was used as a positive control. After 1 minute, fluorescence intensity was read by using Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA) at 450 nm (E_{exc} = 360 nm). The DoF was then calculated as $DoF = [1 - (I_{sample}/I_{control})]$.

2.2.4. Hydrogel preparation

For all the experiments, freeze-dried GelMA was dissolved at the desired concentration (w/v%) into MEM medium at 55°C for 30 minutes. Further, LAP was added as a photoinitiator at a concentration of 2,5 mg/mL. When GelMA and LAP were completely dissolved, the hot solution (55°C) was double filtered previously with 0,45 µm filter and then with 0,22 µm one to sterilize the solution. The photopolymerization was achieved by irradiating the construct with a lamp provided with a 405 nm LED (working distance of 2 cm, power of 52 mW).

3.2.5. Swelling test

To determine swelling behavior, samples were analyzed as reported in literature ¹⁹. Specifically, different GelMA formulations (50 µL) were photopolymerized as described, then, each sample was placed in DPBS at 37 °C overnight. Excess of buffer was removed using a filter paper and the weight of the swollen hydrogel (Ws) was recorded. Samples were then frozen and lyophilized for 24h. In the end, the obtained dried polymer was weighed (Wd). The mass-swelling ratio was calculated as described in the equation below:

$$\text{Swelling ratio (\%)} = \frac{(Ws - Wd)}{Wd} \times 100$$

2.2.6 Dynamo mechanical analysis

The mechanical properties of photocured GelMA hydrogels were assessed by Dynamo Mechanical analysis (DMA). Specifically, cylinders with a diameter of 11 mm and a height of 6 mm were prepared for each GelMA formulation. The tests were performed using in compression mode a Triton Technology DMA (frequency of tests 50 Hz, displacement 50 µm, temperature 37°C).

2.2.7. *In vitro* cell culture

All experiments were performed using lung cancer cell lines and normal fibroblasts. A549 and H1299, two lung adenocarcinoma cell lines, were cultured in BenchStable RPMI 1640 medium with GlutaMAX adding 10 v/v% of FBS and 1 v/v% penicillin/streptomycin. For cytofluorimetric analysis, these cell lines were infected to constitutively expressed GFP protein. MRC-5, a normal lung fibroblast cell line, was maintained in BenchStable MEM medium with GlutaMAX supplemented with 10 v/v% fetal bovine serum, 1 v/v% of MEM non-essential amino acid solution, 1 v/v% of sodium pyruvate and 1 v/v% penicillin/streptomycin. For the experiments, MRC-5 cells were used until passage 10 to avoid senescence. All the cell types were maintained at 37°C in a humidified atmosphere with 5% CO₂.

2.2.8. Conditioned medium experiment

For conditioned medium tests, 1×10^6 A549 or H1299 were cultured in empty MEM (BenchStable MEM medium with GlutaMAX supplemented with 1% penicillin/streptomycin) and were grown for 60h. The day before starting the stimulation with conditioned medium (CM), 5×10^3 MRC-5 were plated into a 96-well plate and starved overnight with empty MEM. Recovered CMs were centrifuged at 2000 rpm for 5 minutes to remove cell debris. Then, 200 μ L of CM were put in each well and MTT analysis were performed after 24h, 48h and 72h.

2.2.9. *In vitro* viability assay

Cell viability was assessed by MTT assay and Live/Dead staining. For MTT analysis in 2D culture, MRC-5 cells were incubated with 200 μ L of 0,5 mg/mL MTT dissolved in MEM for 2 h at 37°C. Then, formazan salts were dissolved in 250 μ L of MTT solvent (10% SDS, 0.01 M HCl in H₂O) and the signal was read after 1h of incubation at 37°C.

For 3D culture, 50 μ L of cell-loaded GelMA were photopolymerized as previously described. After 1 day or 6 days of culture, cell medium was replaced by 200 μ L of 0,5 mg/mL MTT dissolved in MEM and after 4 h of incubation at 37°C, the solution was removed and 200 μ L of DMSO were added to dissolve formazan salts. After 2 h, the plate was analyzed. In both cases, the absorbance was detected by using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA). The optical density was analyzed at wavelength of 570 nm (reference 650 nm). The results were expressed as mean $\pm$ standard deviation and significant differences were measured at a level $p < 0,05$.

For the staining, calcein-AM and PI were diluted according to the manufacturer's instructions. The constructs were washed three times with DPBS, then they were incubated at 37°C with the staining solution for 30 minutes. After three washing steps in DPBS, the fluorescence signal was detected with a microscope (Eclipse Ti2 Nikon, Tokyo, Japan) equipped with a Crest X-Light spinning disk confocal microscope and a Lumencor SPECTRA X light engine. The images were analyzed by Fiji-ImageJ software and the percentage of viable cells was derived as follows:

$$\text{Live cells (\%)} = \frac{(Nt - Nd)}{Nt} \times 100$$

where Nt was the total amount of cells in each image and Nd the number of dead ones.

2.2.10. Cell isolation from GelMA hydrogel

To retrieve cells from GelMA, the hydrogels were digested with a 0,5 mg/mL collagenase solution for 1 hour at 37°C. After complete gel dissolution, cells were washed three times with DPBS, then, the pellet was suspended in the appropriate buffer, according to the analysis required.

2.2.11. Flow cytometry analysis

For this analysis, 50 μ L of cell-loaded GelMA were photopolymerized as previously described. After 1, 6 and 14 days of culture, cells were isolated as described from nine samples in section 2.2.11. and the pellet was suspended in DPBS before the analysis. Flow cytometry was performed with a Guava Easycyte 6-2L flow cytometer (Merck Millipore), collecting for each sample 1×10^4 events, excluding cell debris. Then, data were analyzed by FCS express 6 flow (De Novo Software).

2.2.12. Protein extraction and Western Blot analysis

Protein extracts were obtained by lysing cells, as described in section 2.2.11., using a boiling buffer containing 0,125 M Tris/HCl at pH 6,8 and 2,5% sodium dodecyl sulfate (SDS). Protein concentration was determined by using a BCA protein assay kit, following the manufacturer's instructions. Western blotting (WB) was performed by separating proteins by SDS polyacrylamide gel electrophoresis (PAGE) and electroblotting onto nitrocellulose membranes. Membranes were blocked in 1X Casein solution for 1 hour at RT, and, then incubated with primary antibodies (1 μ g/mL) overnight at 4 °C. Fluorescent secondary antibodies (1 μ g/mL) were incubated for 1 hour at RT and the membrane was analyzed by using Chemidoc Touch Imaging System (Bio Rad). Tubulin was used as a loading control, following the manufacturer's protocol.

2.2.13. Immunofluorescence analysis

For immunofluorescence characterization, cell-laden hydrogels were stained after 14 days of culture. Specifically, the samples were fixed in 4% PAF for 40 minutes. After a washing step, they were incubated for 1 hour in 30 w/v% sucrose solution and, included into PolyFreeze Tissue Freezing Media. Samples were processed on cryotome to create 40 μ m thick sections, mounted on glass slides and kept at -80°C. For staining, sections were washed in DPBS, permeabilized with 0,1 v/v% of Triton X-100 for 10 minutes and blocked with a solution of 5 v/v% Goat serum, 1 w/v% BSA and 0,1 v/v% Tween for 1 hour. For the analysis, three different antibodies were used at specific dilutions: Anti-fibronectin (1 μ g/mL), anti-collagen type 1 (2 μ g/mL) and anti- α -sma (1 μ g/mL). Specifically, primary antibodies were incubated overnight at 4°C and after washing steps, the staining with appropriate secondary antibodies (2 μ g/mL) lasted 1 hour at RT. Nuclei were stained with 0,3 μ M DAPI solution for 10 minutes. At the end, samples were mounted with mowiol and then analyzed by using a microscope (Eclipse Ti2 Nikon, Tokyo, Japan) equipped with a Crest X-Light spinning disk confocal microscope.

2.2.14. RNA extraction and qRT-PCR analysis

Total RNA was obtained by isolating cells by using TRIzol® Reagent. For mRNA detection, RNA was retrotranscribed with High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. qRT-PCRs for mRNA detection were performed with the SYBR® Green PCR Master Mix using a

7900HT Fast Real Time PCR System (Applied Biosystems). The used primers were listed in Table 2.2. Normalization was performed on the expression of β -actin mRNA. The relative expression levels were calculated using the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$)²⁰.

Gene	Forward 5' $\rightarrow$ 3'	Reverse 5' $\rightarrow$ 3'
α -SMA	CCG-ACC-GAA-TGC-AGA-AGG-A	ACA-GAG-TAT-TTG-CGC-TCC-GAA
TGF β	TCG-CCA-GAG-TGG-TTA-TCT-T	TAG-TGA-ACC-CGT-TGA-TGT-CC
FN1	CCA-TCG-CAA-ACC-GCT-GCC-AT	AAC-ACT-TCT-CAG-CTA-TGG-GCT-T
MMP2	GGA-CAC-ACT-AAA-GAA-GAT-GCA-GAA-GT	CGC-ATG-GTC-TCG-ATG-GTA-TTC
Col1a1	TGG-CCC-AGA-AGA-ACT-GGT-ACA-TCA-G	GCT-CTT-CTT-GCA-GTG-GTA-GGT-GAT-G
Actin	CCT-GGC-ACC-CAG-CAC-AAT	GCC-GAT-CCA-CAC-GGA-GTA-CT

Tab. 2.2: List of primers used for qRT-PCR.

2.3. Results and Discussion

2.3.1. GelMA synthesis and DoF evaluation

GelMA was selected as a matrix for NSCLC's scaffold because of its known biocompatibility, the presence of a cell adhesive site and the low toxicity after the polymerization reaction¹⁴. GelMA synthesis was previously described¹³. In brief, 10% of the gelatin was dissolved into DPBS and different amounts of MA were slowly added to the solution to introduce methacryloyl groups on the reactive amine and hydroxyl groups of the gelatin. This modulation was directly correlated to different degrees of functionalization (DoF) of the matrix. After, the reaction was stopped, the solution was dialyzed against deionized water to remove all unreacted species and freeze-dried until a soft foam was obtained. The scheme of reaction and the successive polymerization are shown in Fig. 2.1a. The methacryloyl substitution of obtained formulations was analyzed by using fluoraldehyde o-phthalaldehyde (OPA) reagent, which reacts with primary amines²¹. Specifically, the signal derived from OPA assay is directly correlated to the amount of free primary amines, which is inversely correlated to the quantity of methacryloyl groups added to the gelatin backbone. As shown in Fig. 2.1b, mean DoFs were 63%, 70%, 78% and 97% for Low, Medium, High and Very High solutions, respectively. For this analysis, unmodified gelatin was used as a control and the measurements were repeated at least three times. DoF determination was essential for the biophysical characterization of the materials. Indeed, the amount of methacryloyl substitution is directly correlated to the mechanical properties of the resulting hydrogel, the crosslinking density and the pore size²². Furthermore, stiffness regulation is directly correlated to cell behavior²³, hence DoF modulation could be related to cell viability and effective tumor model establishment.

Next, LAP was selected as photoinitiator. Indeed, it was characterized by high water solubility and reduced cell toxicity¹⁷. Specifically, it belonged to type I photoinitiator group in which the process of free-radical

photopolymerization starts with the homolytic dissociation of the photoinitiator molecule under UV/visible light. Importantly, unlike the widely used Irgacure 2959, LAP is mildly activated also in the visible light range ($\lambda = 405$ nm)²⁴. The mechanism is briefly described in Fig. 2.1c.

Then, the sterilization process was optimized. From the literature, it is known that this step has a strong impact on GelMA mechanical properties and printability²⁵. Since UV-sterilization of the foam did not result in promising results and could induce slight polymerization, warm GelMA solutions were filtered using 0,45 μ m and 0,22 μ m polyethersulfone (PES) filters under laminar flow in the biological hood. To correctly characterize the physical features of GelMA formulations, all the analyses were performed after the sterilization process.

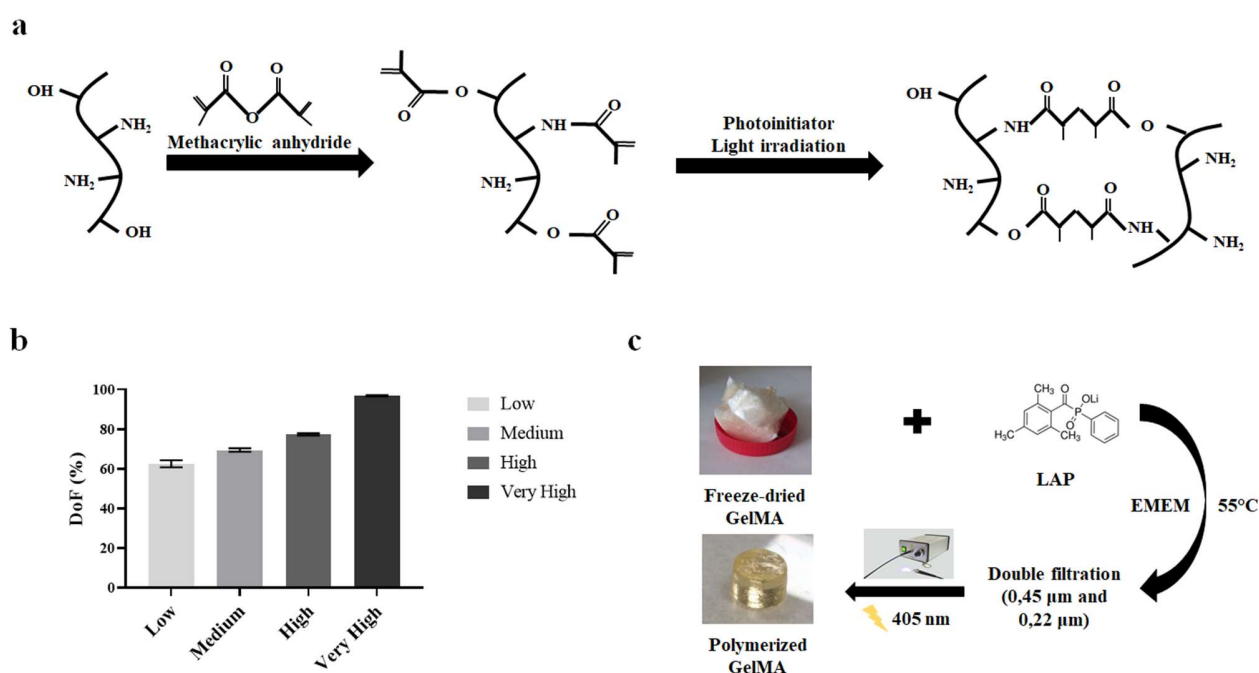


Fig. 2.1: a) Scheme of the reactions that occurred during GelMA synthesis and photopolymerization. b) Methacryloyl substitution of different GelMA formulations determined by fluorolaldehyde o-phthalaldehyde assay. Results were shown as mean $\pm$ SD. c) Scheme of GelMA preparation and polymerization.

2.3.2. Physical and mechanical characterization of different GelMA formulations

Since the goal of the project was the optimization of a biocompatible matrix, which could be easily handled, GelMA low formulation was not considered for further analysis because of its low DoF, which was directly correlated to hydrogel compressive modulus²². Indeed, the final hydrogel was quite soft and could be easily destroyed during handling after a few hours of swelling in cell culture medium (Fig. 2.2a) and, most importantly, cell sedimentation was detected when the hydrogel was loaded (Fig. 2.2b).

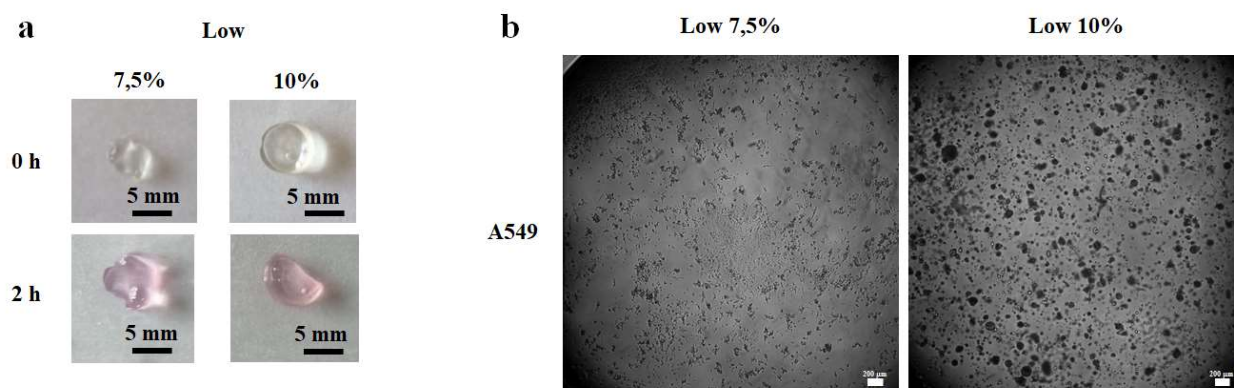


Fig. 2.2: a) Representative images of cylinder of GelMA Low at the concentration 7,5 w/v% and 10 w/v% before and after 2 h of swelling in cell culture medium b) Brightfield images of A549 cells seeded in GelMA Low at different concentration (i.e. 7,5 and 10 w/v%) and cultured for 6 days. Scale bar: 200 μ m.

GelMA Medium, High and Very High were dissolved in culture medium at different percentages and samples were photopolymerized in a 96-well plate. At this point, the effect of 2 h and 24 h of swelling was assessed and as expected, all the samples, with the exclusion of GelMA Medium at the concentration of 7,5 w/v%, remained intact after 24 h of immersion in cell culture medium (Fig. 2.3), suggesting the feasibility of further investigation of their potential as matrix for 3D culture

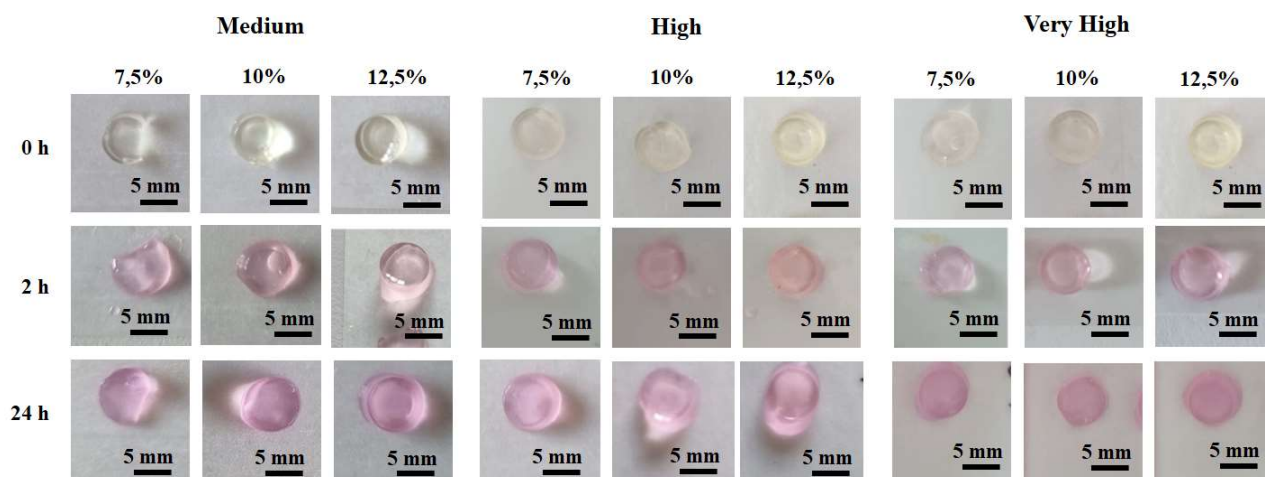


Fig. 2.3: a) Representative images of cylinder of GelMA Medium, High and Very High at the concentration 7,5 w/v%, 10 w/v% and 12,5 w/v% before and after 2 h and 24 h of swelling in cell culture medium.

Further, the capability of the hydrogel to absorb water was characterized through a swelling analysis. Indeed, it is known that the swelling ratio is strongly influenced by the crosslinking density. Hence, as expected, an increase in GelMA concentration was associated with decreasing in swelling capability and GelMA Medium was characterized by the higher percentage of swelling compared to High and Very High, which showed similar behavior (Fig. 2.4a).

The next analysis, was the evaluation of hydrogel stiffness. As reported in literature, stiffness is an important properties of a hydrogel, so that the mechanical properties of a hydrogel had a strong impact on cell behavior²³. Elastic moduli of different GelMA hydrogels were characterized by dynamo mechanical analysis and, as shown in Fig. 2.4b, the highest storage modulus resulted in hydrogel with the higher concentration of GelMA. In detail, GelMA Medium presented the lowest elastic moduli, whereas the behavior of High and Very High was quite similar, suggesting that the GelMA percentage had a higher impact on hydrogel stiffness rather than DoFs. Since it has been shown that lung cancer is stiffer than the surrounding healthy tissue²³, GelMA formulations with higher DoFs and concentration have been expected, so to be more suitable for the establishment of adenocarcinoma models.

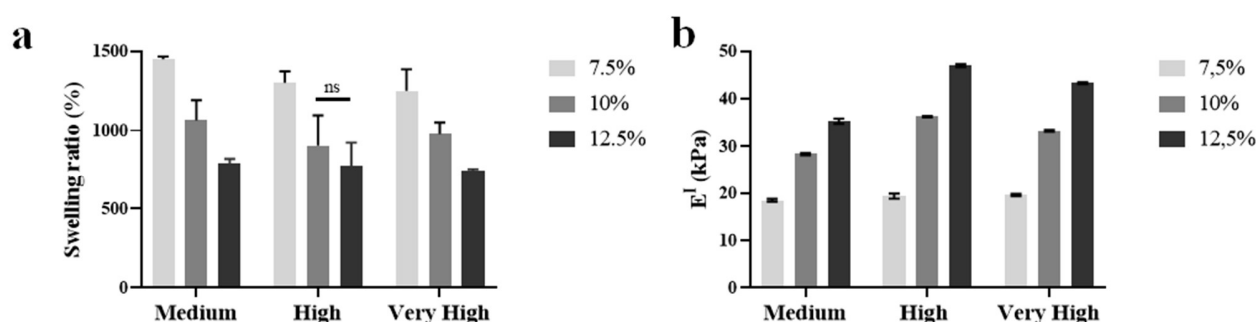


Fig. 2.4: a) Equilibrium swelling and b) elastic modulus of different formulation of GelMA hydrogels. The results were shown as mean±SD.

2.3.3. Analysis of the influence of tumor cells' conditioned medium on MRC-5 proliferation rate

Since the goal of the work was the study of the interactions between cancer cells and the stroma, the crosstalk between lung adenocarcinoma cell lines (i.e. A549 and H1299) and normal lung fibroblasts (MRC-5) was investigated. It was previously shown that MRC-5 proliferation was enhanced by tumor cells²⁶, and moreover, their metabolic activity was influenced by A549 and H1299 through paracrine signaling²⁷.

In this thesis, the response of fibroblasts after stimulation with a culture medium conditioned (CM) by the tumor cells without the influence of the matrix was analyzed. Specifically, differences in proliferation rate were detected by MTT assay following the experimental setting shown in Fig. 2.5a. Precisely, not supplemented fibroblast's medium was conditioned by A549 or H1299 cultured in 2D for 60h, then the effect on MRC-5 was monitored at

different time points (i.e. 24h, 48h and 72h) using the signal at the beginning of the experiment (i.e. 0h) as starting point. Interestingly, factors released by A549 strongly increased fibroblast proliferation and the differences with the control (i.e. cells grown with not-conditioned media) were statistically significant at any time point. Instead, this effect was detectable only after 24h in MRC-5 grown with H1299 CM, indicating a transient effect on fibroblast proliferation (Fig. 2.5b). To conclude, these results suggested that crosstalk between the selected cell lines happened during the culturing, but the effect on proliferation was more relevant for A549 whereas H1299's secreted factors had a mild interaction. Thus, the investigation was moved to 3D co-culture by using different GelMA formulations.

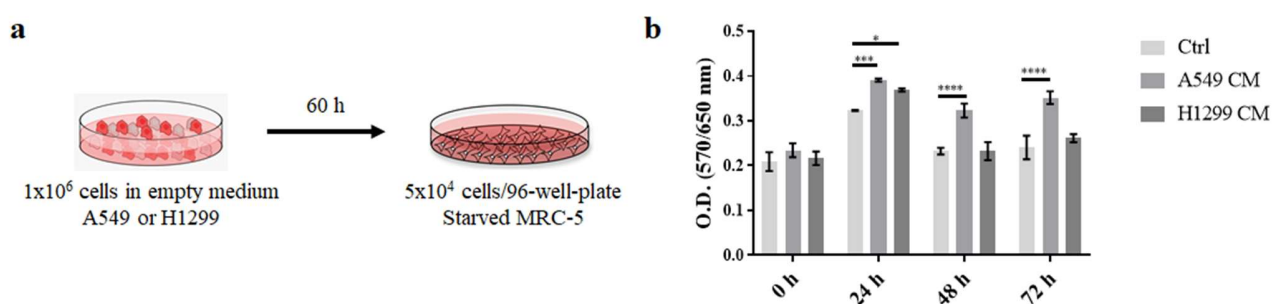


Fig. 2.5: a) Scheme of conditioned-medium (CM) experiment set up. b) Starved MRC-5 were grown with not-conditioned medium (Ctrl) or with conditioned ones (A549 CM or H1299 CM). Proliferation rate was analyzed by MTT assay at 0h, 24h, 48h and 72h after the beginning of the conditioning. Results were shown as mean±SD. *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001

2.3.4. Optimization of cell density into GelMA hydrogel

The next step was the evaluation of the appropriate cell density to establish a 3D model. Indeed, cell amount and cell-cell distance had an impact on cell viability, behavior and survival, especially if the hydrogel would be later used for bioprinting²⁸. At the beginning of the study, GelMA Medium at 10 w/v% in MEM was chosen as matrix for this preliminary evaluation and three different cell concentrations were assessed (i.e. 1x10⁶, 1,5x10⁶ and 2x10⁶ cells/mL). Specifically, GelMA Medium was loaded with A549, H1299 or MRC-5 and the effect of the hydrogel on cell proliferation rate was measured after 1 and 6 days of culture. As shown in Fig. 2.6a, the proliferation of A549 was strongly enhanced at day 6, especially for 1x10⁶ and 1,5x10⁶ density. Interestingly, on day 1 the differences between cell amounts were significant, while at the endpoint the signals were comparable. These results suggested that the activation of A549 was higher at lower cell density. On the other hand, H1299 did not present a strong increase in proliferation rate after 6 days of culture, except for 1,5x10⁶ density (Fig. 2.6b). The scenario strongly changed when MRC-5 cells were analyzed. Indeed, fibroblasts showed a significant signal decrease over time (Fig. 2.6c), that could be explained by the insurgence of GelMA-related toxicity or slowdown of their metabolic activity and cell cycle activation. Taken together, these results demonstrated variability in response to GelMA inclusion, depending on cell types and amounts. Overall, 1,5x10⁶ density showed better results

for A549 and H1299, specifically an increase of 61% and 48% in MTT signal between day 1 and day 6. Moreover, these preliminary analyses demonstrated the influence of GelMA Medium hydrogel on different cell types. Hence, deep evaluation of different formulations and concentrations must be done to find the best conditions to promote cell survival and viability.

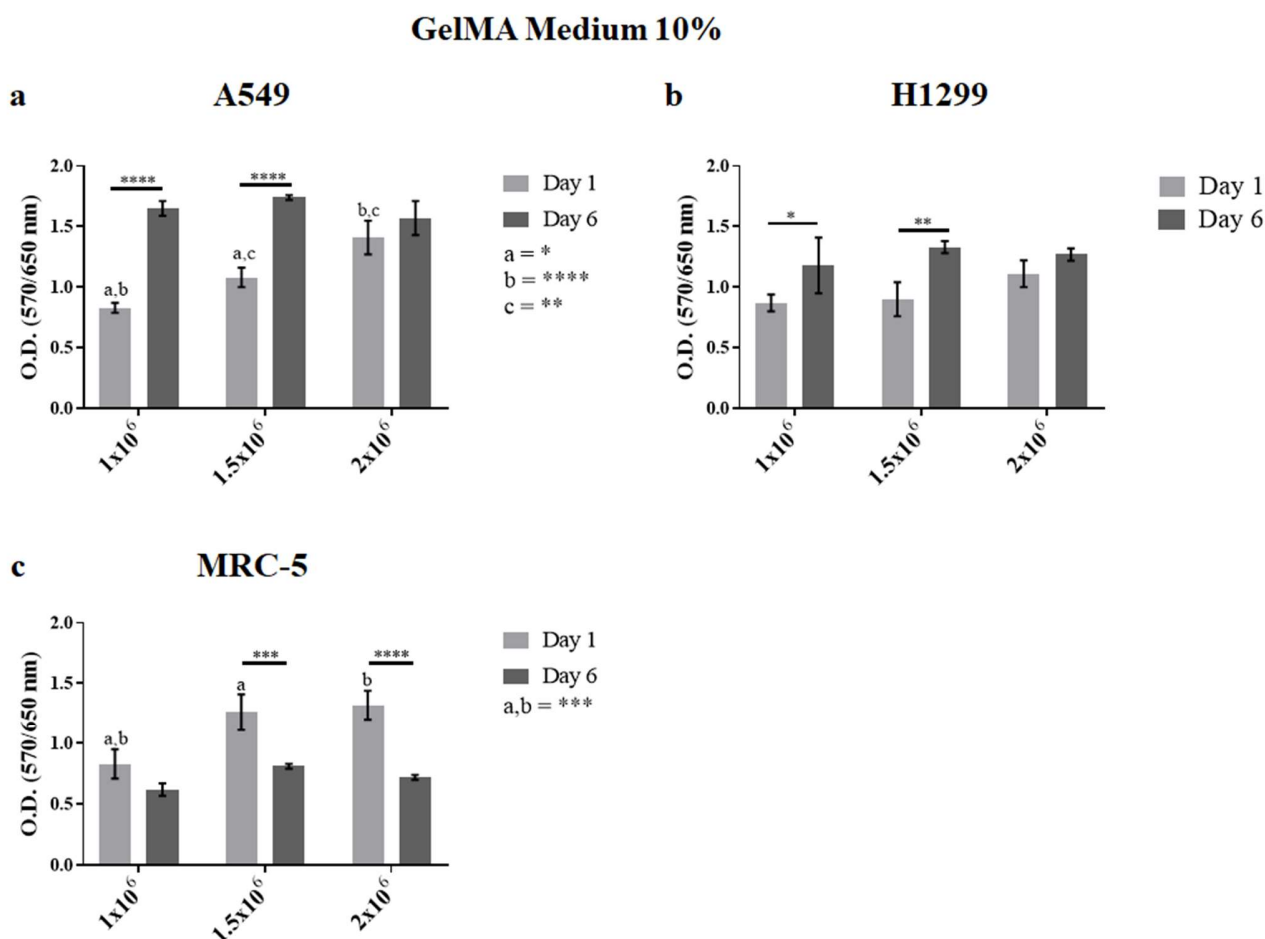


Fig. 2.6.: MTT assay. A549 (a), H1299 (b) or MRC-5 (c) were dispersed into GelMA Medium at 10 w/v% in MEM. MTT analysis was performed after 1 day or 6 days of culture. Results were presented as mean $\pm$ SD. *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001

2.3.5. Analysis of cells proliferation rate in different GelMA formulations

To systematically analyze GelMA formulations, precise culture conditions had to be set in order to choose the best ones for the establishment of NSCLC models. Aiming to study the effect of the microenvironment on cancer progression, co-culture of tumor cells and fibroblasts should be designed. Importantly, the presence of these two populations influenced cell viability ²⁹, hence two ratios between cell types were selected, as described in the

experimental set-up shown in Tab. 2.3a. Further, mono-cultures of A549, H1299 and MRC-5 were used as control. Moreover, the analysis focused on two models named A549 or H1299 according to the tumor cell types used. Then, several GelMA DoFs and concentrations in MEM (w/v%) were analyzed to find the most suitable for each model (Tab. 2.3b).

a	Culture condition	Cell types amount (%)	
		Tumor cells	Fibroblasts
	Tumor cells	100 %	0 %
	Fibroblasts	0 %	100 %
	1:1	50 %	50 %
	1:2	33,3 %	66,7 %

b	Model	GelMA DoF	GelMA concentration in MEM		
			7,5 w/v%	10 w/v%	12,5 w/v%
A549		Medium	X	X	
		High	X	X	
		Very High	X	X	
H1299		Medium	X	X	
		High	X	X	X
		Very High	X	X	X

Tab.2.3.: Mono-culture and co-culture set up (a) and description of tested hydrogel formulations indicated by an “X” with different DoFs and concentrations (b)

The differences between the proposed hydrogels were analyzed by MTT assay after 1 day and 6 days of culture to assess variations in proliferation rate (Fig. 2.7, 2.8).

A549 model showed promising results especially for GelMA at 10 w/v% in MEM (Fig. 2.7). To select the suitable formulations among GelMA 10 w/v% with different DoFs, the proliferation rate of the co-culture was investigated. Specifically, in 1:1 ratio, the cells showed an increase of the signal from day 1 to day 6 of 20%, 88% and 66% in Medium, High and Very High, respectively. Moreover, in 1:2 co-culture ratio, the raises were of 34%, 63% and 113%. GelMA Medium was less adequate in sustaining a good level of cell growth in the co-culture, so GelMA High and Very High at 10 w/v% in MEM were selected to be further analyzed for the establishment of A549 model (Fig. 2.7 b,d,f).

Then, the investigation moved to **H1299 model**. Interestingly, GelMA at 7,5 w/v% and 10 w/v% in MEM did not show promising results in terms of an increase in proliferation rate (Fig. 2.8 a-f). This behavior could be explained by the differences that occur between the two cell types. In fact, although A549 and H1299 were both epithelial cells from lung adenocarcinoma, they differed in terms of molecular characteristics and actions. H1299 showed higher invasive potential compared to A549 which was justified by their epithelial-to-mesenchymal transition³⁰. Moreover, this invasion capability was promoted by different proteins that digested the ECM in the tumor microenvironment. Among those, Matrix Metalloproteinases (MMPs) were a group of enzymes able to degrade all components of the ECM, promoting tumor invasion and progression³¹. Since gelatin presented a site recognized and cleaved by MMPs³², we expected consistent degradation of GelMA and cell escape from the hydrogel, when loaded with tumor cells with relevant invasion capability. These properties could explain the

absence of signal increase for H1299 after 6 days of culture. Thus, more concentrated GelMAs were tested. GelMA High and Very High at 12,5 w/v% in MEM showed a great increase in proliferation rate over time (Fig. 2.8 g,h). Specifically, the increases in 1:1 ratio were of 130% and 93% and in 1:2 condition of 120% and 110% for High and Very High, respectively. Thus, these conditions were selected for further analysis for H1299 model.

A549 model

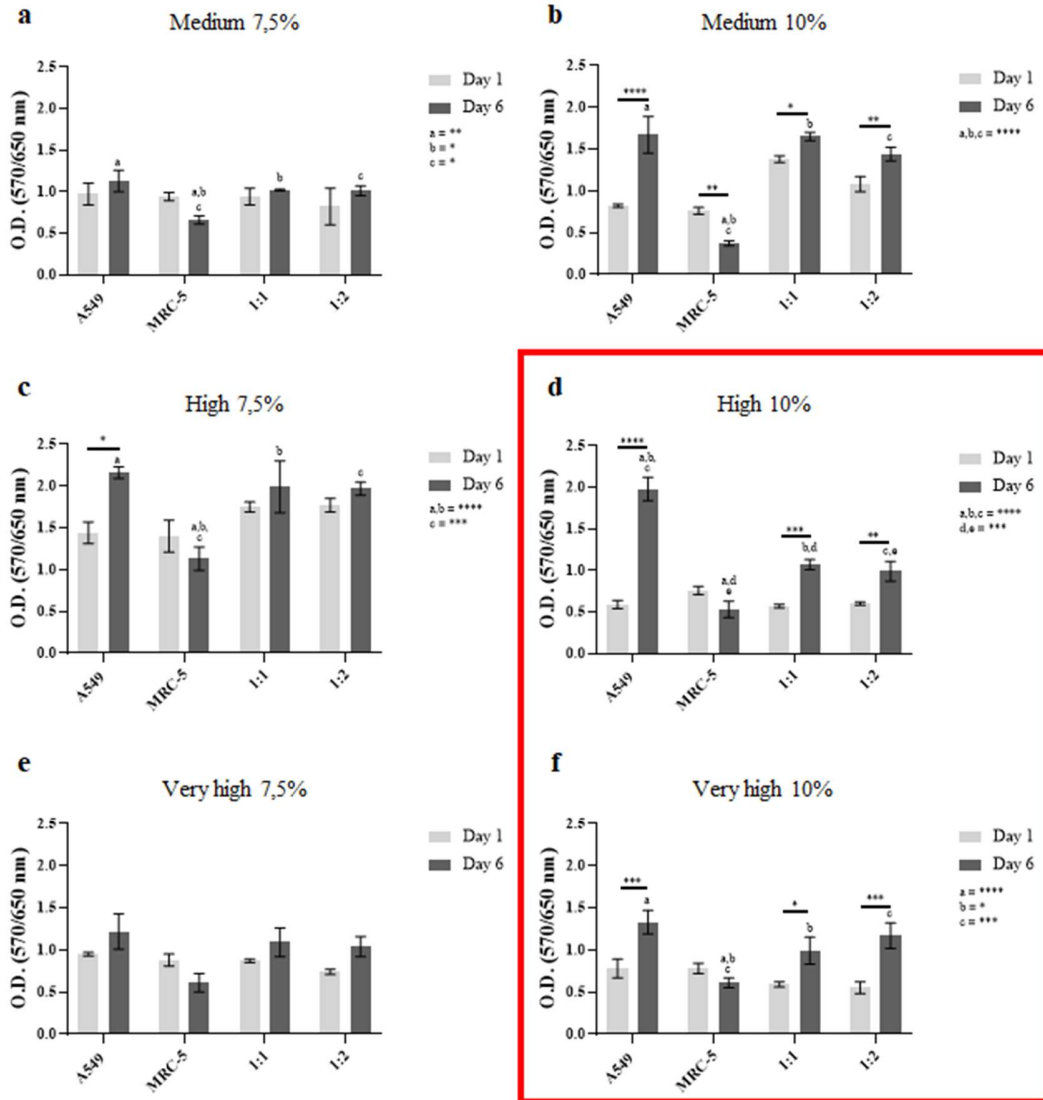


Fig. 2.7: MTT assay. A549 and MRC-5 were dispersed into GelMA with different DoFs: Medium (a,b), High (c,d) and Very High (e,f). Hydrogels were prepared at 7,5 w/v% in MEM (a,c,e) or at 10% (b,d,f). The signal was measured after 1 day or 6 days of culture. Tests were performed at least three times and the results were presented as mean±SD. *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001

H1299 model

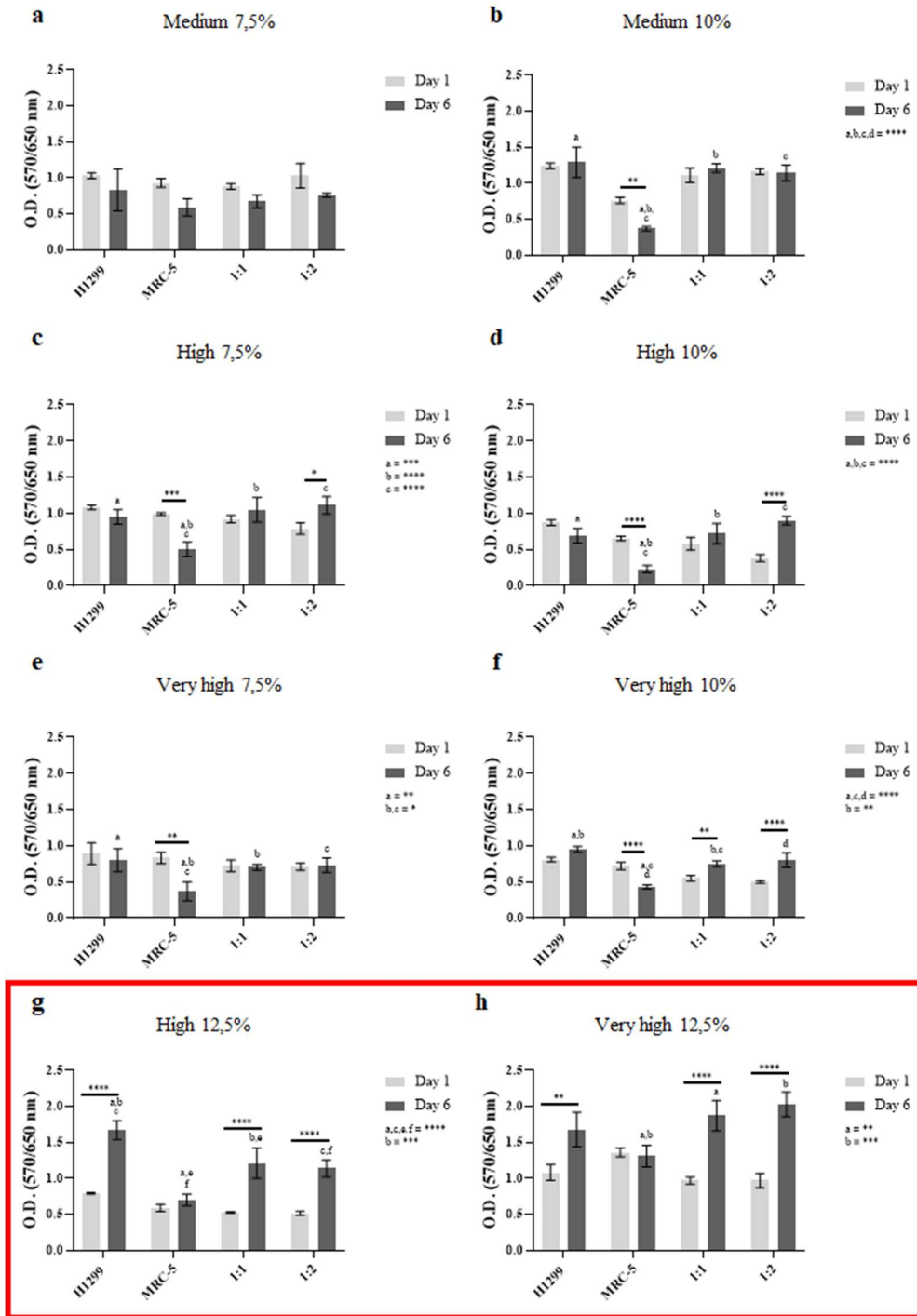


Fig. 2.8: MTT assay. H1299 and MRC-5 were dispersed into GelMA with different DoF: Medium (a,b), High (c,d,g) and Very High (e,f,h). Hydrogels were prepared in MEM at 7,5 w/v% (a,c,e), at 10 w/v% (b,d,f), or 12,5 w/v% (g,f). The signal was measured after 1 day or 6 days of culture. Tests were performed at least three times and the results were presented as mean±SD. *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001

2.3.6. Cell viability level in A549 and H1299 models

MTT analysis showed how cells proliferate in the hydrogel, but this test did not give any information about cell viability. To investigate it and confirm the suitability of our matrices for the development of NSCLC model, Calcein AM and PI staining was used. In details, the constructs were grown for 14 days and the viability was tested after 6 and 14 days of culture. As shown in Fig. 2.9a, the viability level in A549 model was higher than 90% and 80% after 6 or 14 days of culturing, respectively. Although a slight increase in PI signal was observed after 14 days, the high viability value means that the matrices did not induce strong cell death over time. Promising results were obtained also for H1299 model, in which the viability was always higher than 90% (Fig. 2.9b). Once more, these data demonstrated the feasibility of using these formulations for the establishment of long-term NSCLC cultures.

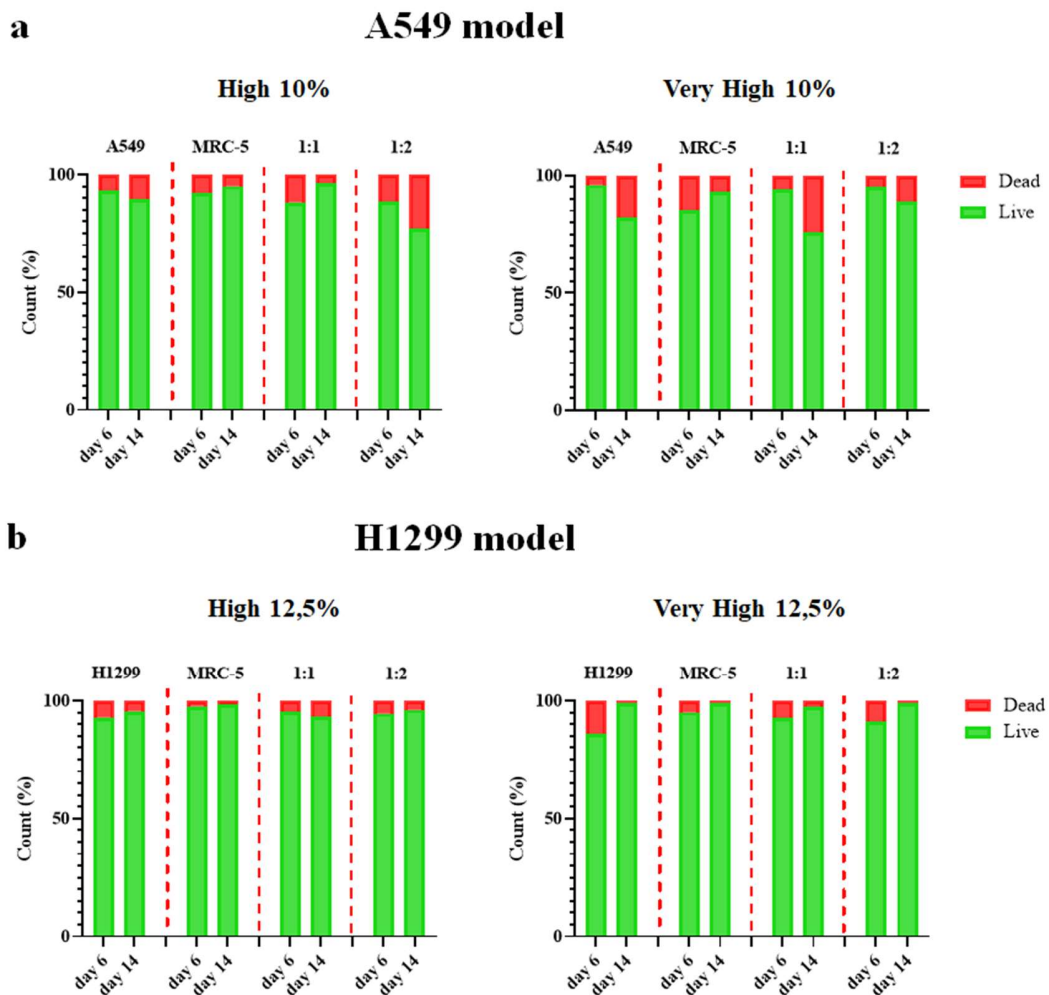


Fig. 2.9: Live&Dead assay. A549 (a) or H1299 (b) model viability was assessed by Calcein AM and PI staining. The tests were performed after 6 days or 14 days of culture. Results were presented as percentage of viable (green) or dead (red) cells.

2.3.7. Assessment of the maintenance of MRC-5 in the co-culture

A strong limitation of the previous analyses was the impossibility to distinguish the contribution of the two cell types, precisely tumor cells and fibroblasts, in the co-culture. Interestingly, MTT analysis clearly demonstrated that, when MRC-5 cells were grown as mono-culture, they did not proliferate in the hydrogels despite the changing of DoF and GelMA concentration (Fig. 2.7 and 2.8). Despite that, Calcein AM and PI staining showed good viability (Fig. 2.9). So, their contribution to the co-culture could be low or absent. Thus, the open issue was if fibroblasts were maintained over time in the co-culture or if their contribution was lost. To assess this, A549 and H1299 were infected to constitutively express histonic protein H2B fused with the green fluorescent protein (GFP), in order to distinguish tumor cells (A59 or H1299) from non-fluorescent MRC-5. To retrieve cells from the hydrogel, GelMA was digested by collagenase enzyme, thanks to the presence of cleavable sites³³. Then, samples were tested by cytofluorimetric analysis to estimate the amount of fluorescent and non-fluorescent cells in each construct. Importantly, mono-culture of tumor cells (A59 or H1299) and MRC-5 were used as a positive and negative control, respectively (data not shown). As depicted in Fig. 2.10, both the cell types were maintained over time in the co-cultures. Interestingly, the percentage of the two populations was preserved from day 1 to day 14, showing the absence of tumor cell increase at the expense of the fibroblasts. Taken together, these results demonstrated the feasibility of using the selected conditions as a scaffold for the establishment of co-culture mimicking the composition of NSCLC stroma.

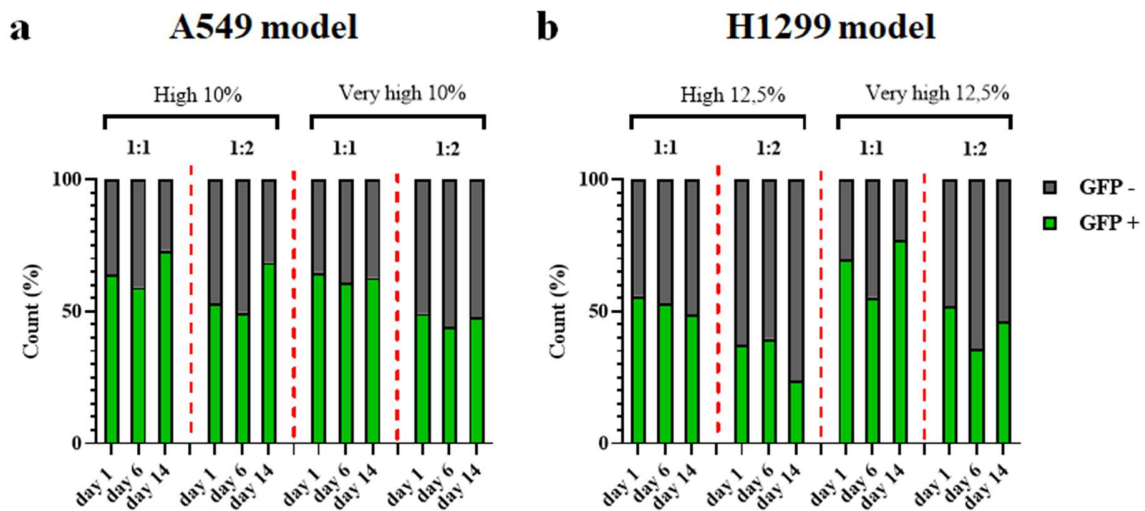


Fig. 2.10: Cytofluorimetric analysis. GFP+ A549 or GFP+ H1299 and MRC-5 were dispersed into GelMA. Fluorescence of cells recovered from co-culture constructs of A549 (a) or H1299 (b) model was analyzed after 1 day, 6 days or 14 days of culture. Results were presented as percentage of fluorescent (green) or non-fluorescent (grey) events.

2.3.8 Collagen I, Fibronectin and α -sma protein expression analysis

To test fibroblasts contribution and the interaction between the two cell populations in the co-culture, western blot analysis was performed in the selected scaffold. Alpha-smooth muscle actin (α -sma) is usually expressed in cancer associated fibroblasts (CAFs), which in turn promotes fibrosis. Particularly, changes linked to α -sma expression and fibrosis promote tumor proliferation, suppression of the immune response and drug resistance ³⁴. In addition, collagens are the main content of the extracellular matrix, where collagen type I is formed by three coiled subunits that form fibrils ³⁵ and its deposition is linked to lung adenocarcinoma progression ³⁶. Since their importance in the tumor microenvironment, Collagen type I and Alpha-smooth muscle actin expressions was assessed by western blot analysis in the optimized constructs. Interestingly, a strong increase was observed for both proteins in fibroblast mono-culture, except for MRC-5 cultures in Very High 12,5 w/v%, in which Collagen type I expression remained low also after 14 days (Fig. 2.11 a,b). These results suggested an activation of the fibroblasts during the 3D culture. Surprisingly, this modulation was almost completely lost in the co-culture for all the conditions (Fig. 2.11 a,b). Since MRC-5 presence was demonstrated by cytofluorimetric analysis (Fig. 2.10), this down modulation could be caused by the interactions that occurred between fibroblasts and tumor cells when cultured together.

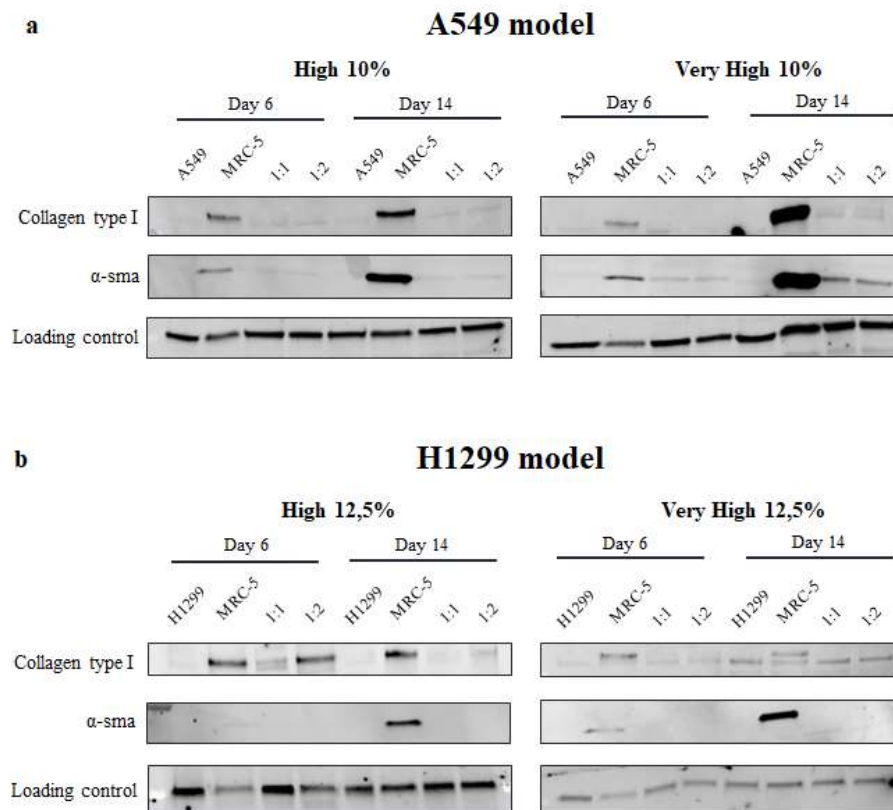


Fig. 2.11: Western Blot analysis. A549 or H1299 and MRC-5 were dispersed into GelMA. Protein were extracted from cells recovered from mono- or co-culture constructs of A549 (a) or H1299 (b) model after 6 or 14 days of culture. Western Blot analysis were performed for assessing Collagen Type I and α -sma expression level. Tubulin was used as loading control.

To further demonstrate the expression of α -sma and Collagen type I protein on MRC-5 culture, immunofluorescence analysis was performed on samples cultured for 14 days. In all conditions, α -sma was clearly detectable in MRC-5 mono-culture confirming the previous western blot analysis (Fig. 2.12-15). Interestingly, Collagen Type I signal was observable also in A549 mono-culture and in the co-cultures (Fig. 2.12, 2.13). On the other hand, it was almost absent in H1299 model apart from MRC-5 mono-cultures, as expected according to Western Blot analysis (Fig. 2.14, 2.15).

Among the molecules involved in the modulation of the tumor niche, Fibronectin plays a crucial role in promoting invasion, migration and angiogenesis ³⁷. Thus, its expression was assessed by immunofluorescence analysis in both models. As shown in Fig. 2.16-19, its production was greatly enhanced in MRC-5 in the mono-culture, while it was absent in the co-culture, suggesting again a different modulation of fibroblast' activity, when cultured in the presence of tumor cells. These results were quite unexpected, because they suggested that the presence of the tumor cells inhibited the capability of the fibroblasts to produce components of the ECM useful for the creation of a more pervasive microenvironment.

A549 model

High 10%

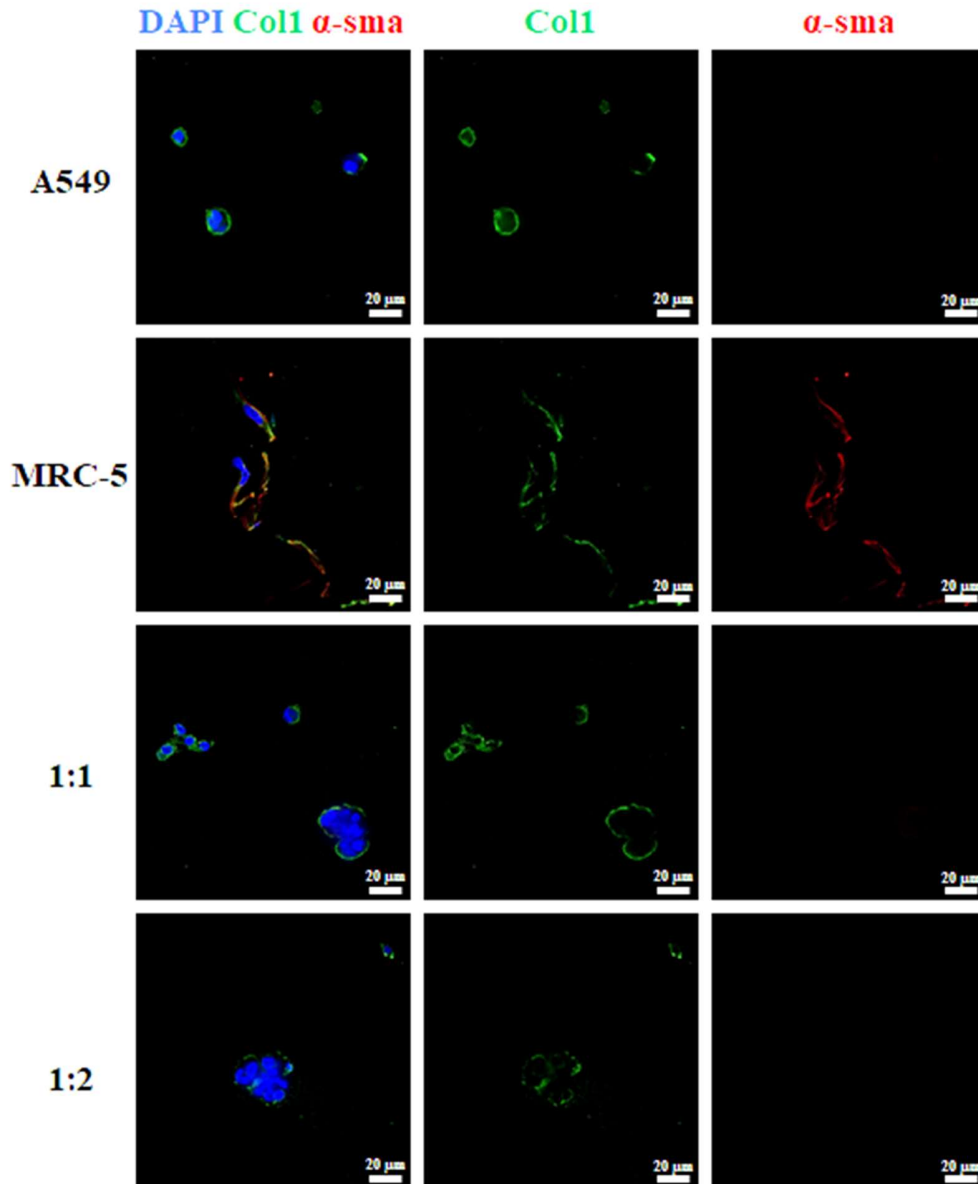


Fig. 2.12: Immunofluorescence analysis on A549 model cultured in GelMA High 10%. The staining was performed after 14 days of culture. Collagen type I (Col1) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

A549 model Very High 10%

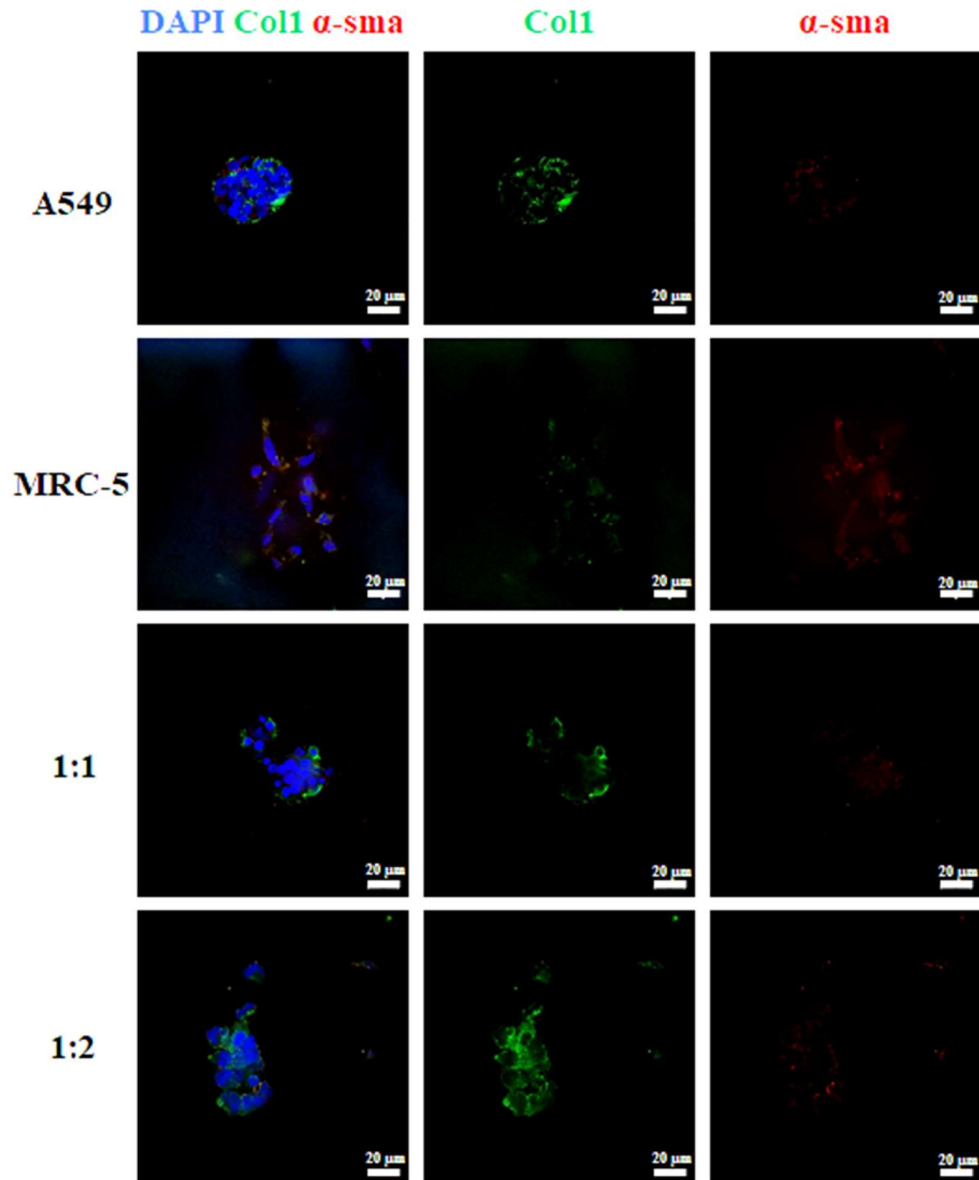


Fig. 2.13: Immunofluorescence analysis on A549 model cultured in GelMA Very High 10%. The staining was performed after 14 days of culture. Collagen type I (Col1) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm, Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

H1299 model High 12,5%

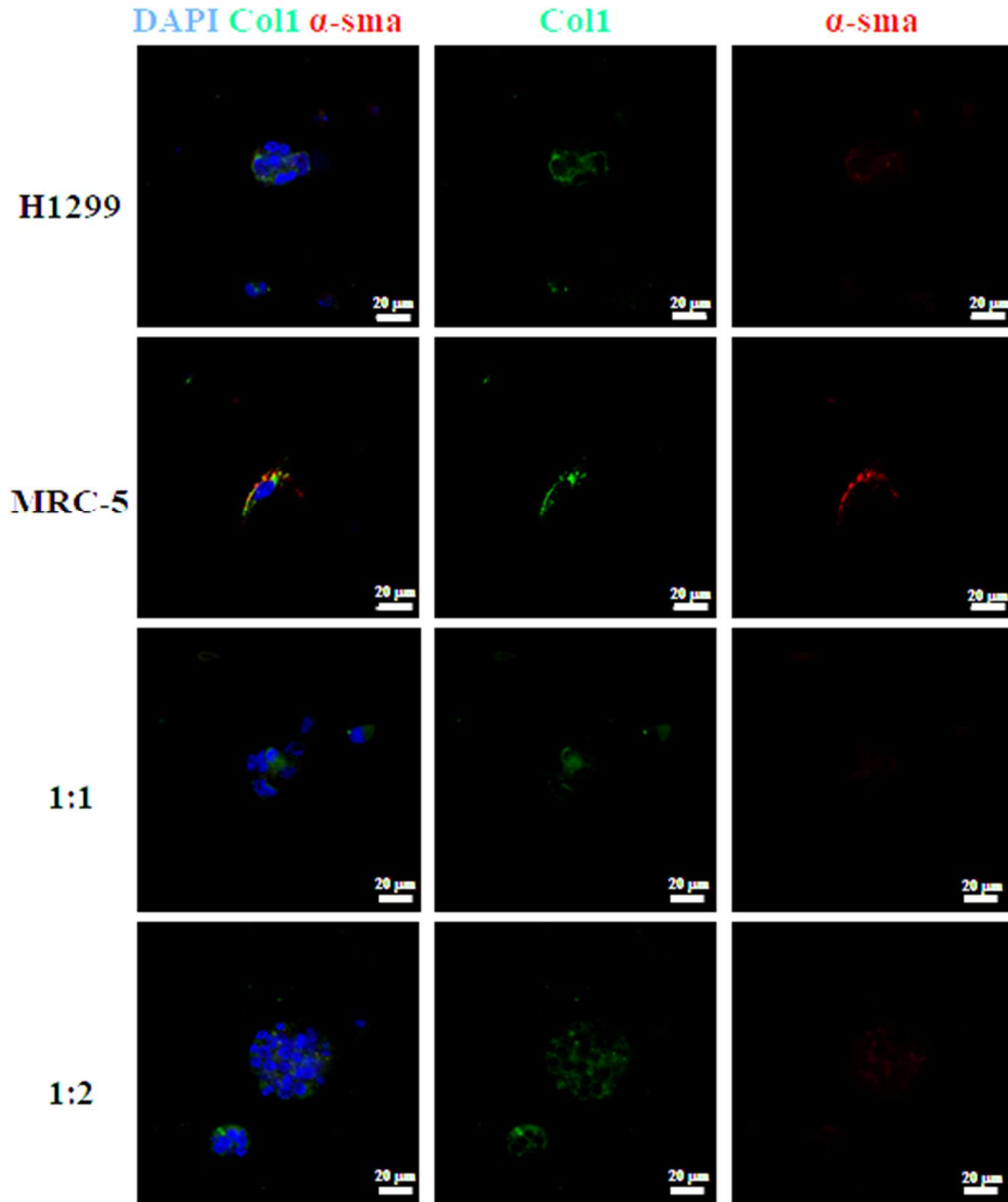


Fig. 2.14: Immunofluorescence analysis on H1299 model cultured in GelMA High 12,5%. The staining was performed after 14 days of culture. Collagen type I (Col1) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

H1299 model Very High 12,5%

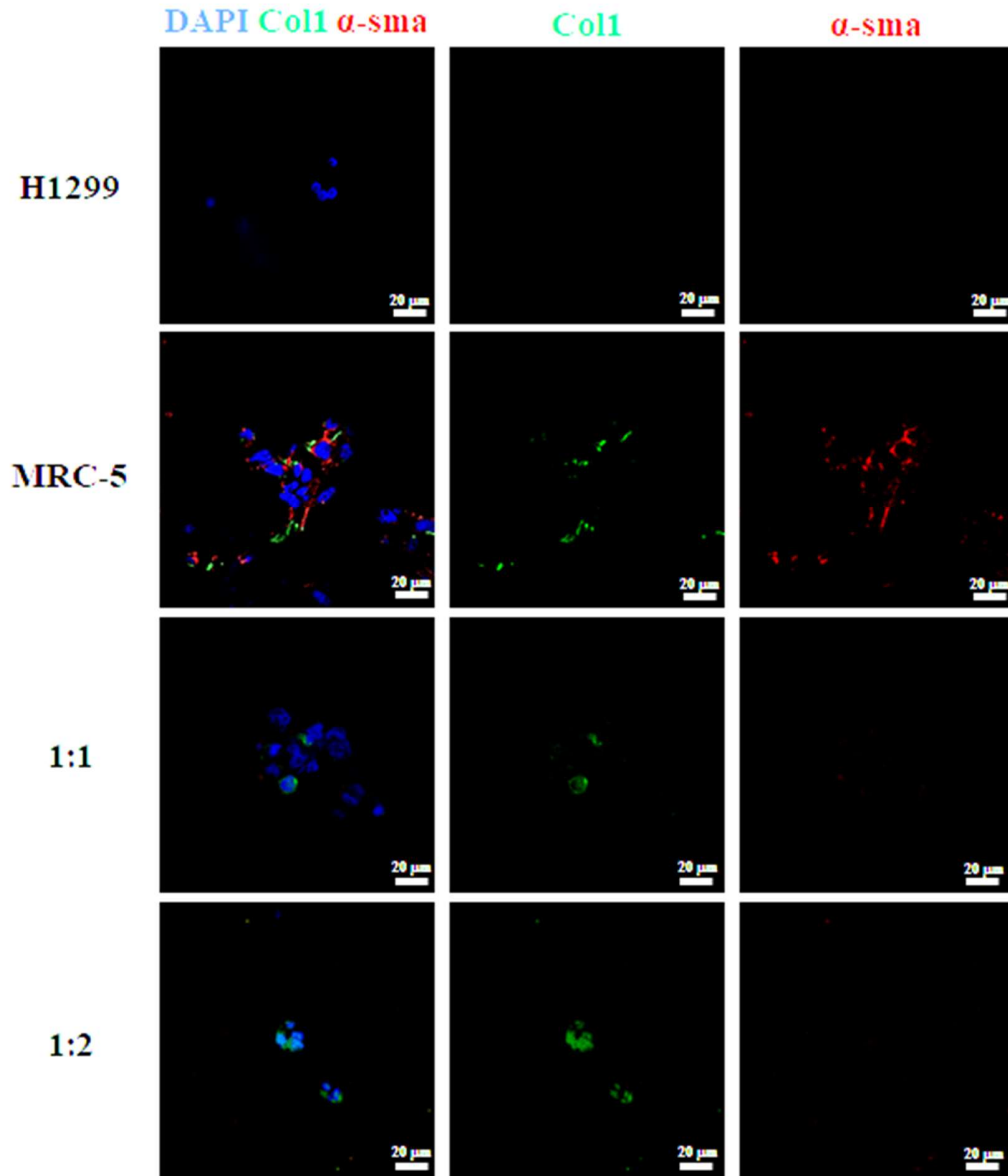


Fig. 2.15: Immunofluorescence analysis on H1299 model cultured in GelMA Very High 12,5%. The staining was performed after 14 days of culture. Collagen type I (Col1) and α-sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

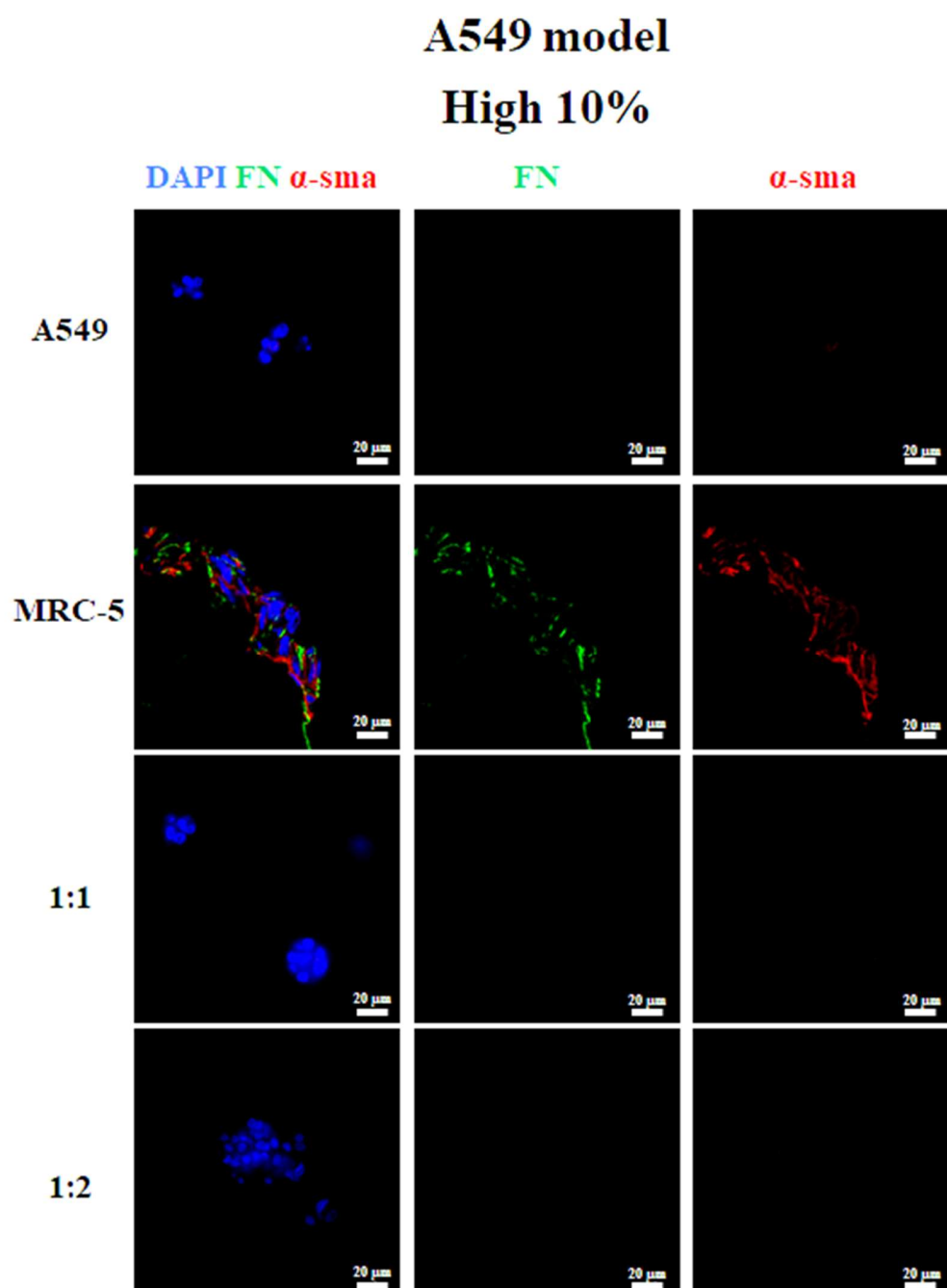


Fig. 2.16: Immunofluorescence analysis on A549 model cultured in GelMA High 10%. The staining was performed after 14 days of culture. Fibronectin (FN) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

A549 model Very High 10%

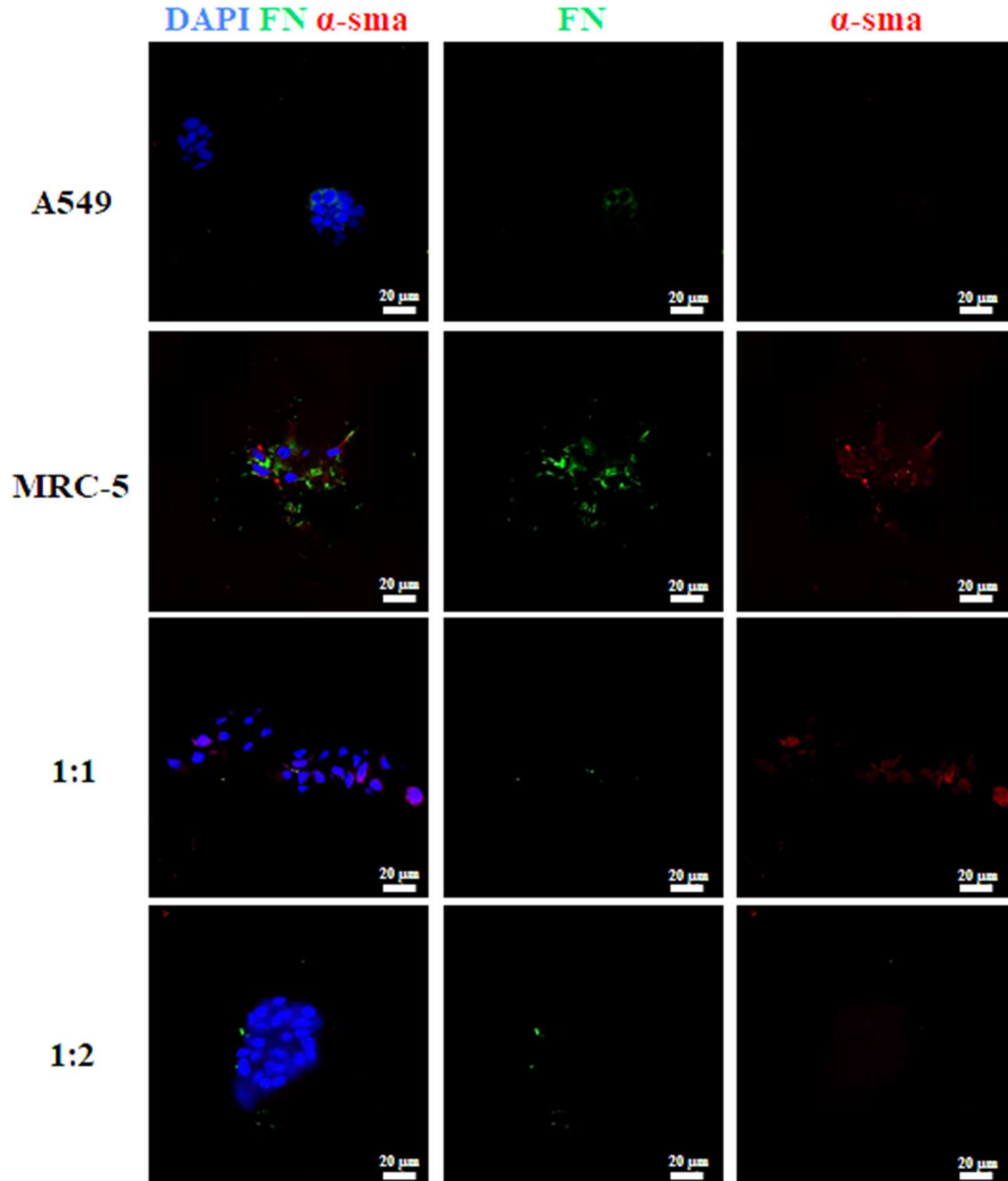


Fig. 2.17: Immunofluorescence analysis on A549 model cultured in GelMA Very High 10%. The staining was performed after 14 days of culture. Fibronectin (FN) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

H1299 model High 12,5%

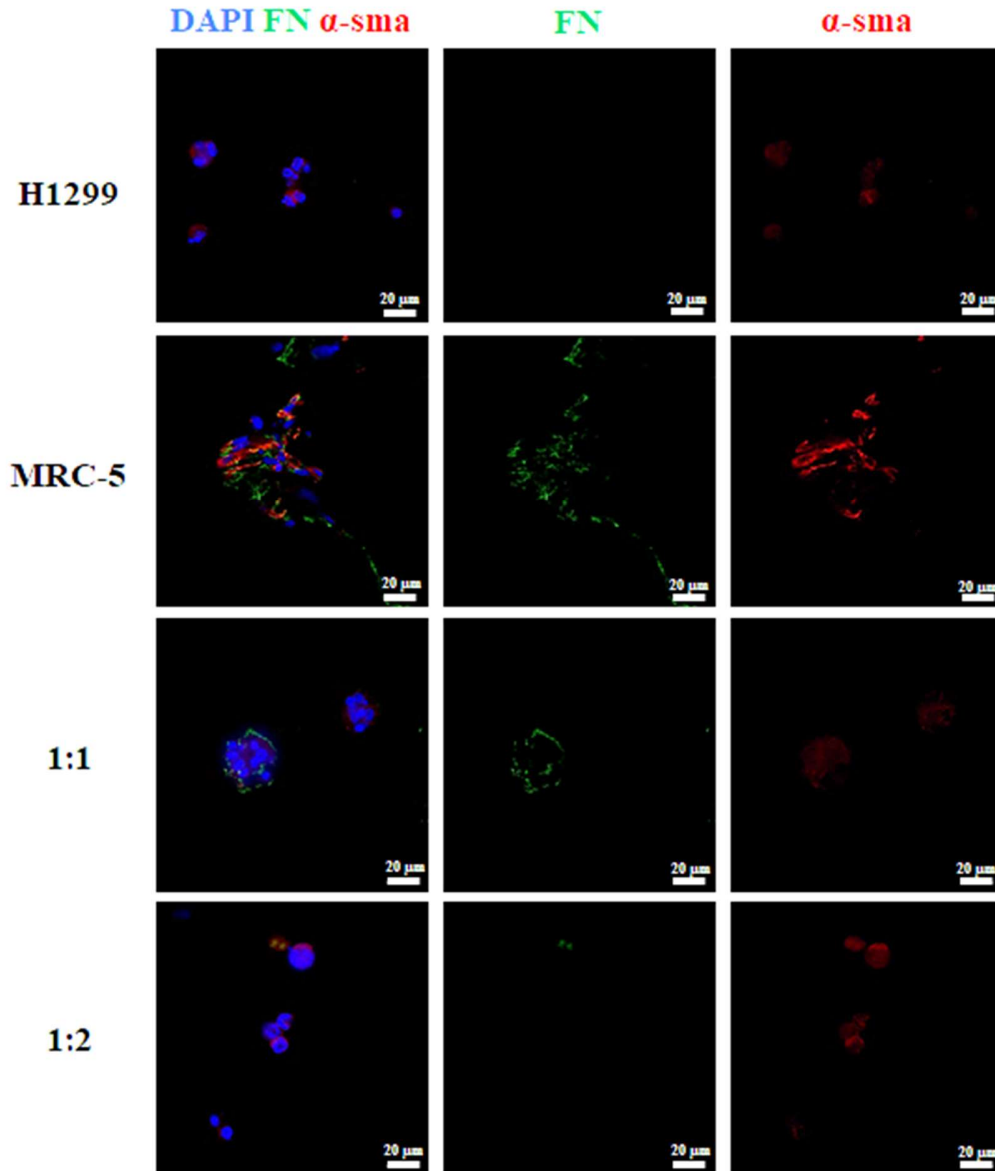


Fig. 2.18: Immunofluorescence analysis on H1299 model cultured in GelMA High 12.5%. The staining was performed after 14 days of culture. Fibronectin (FN) and α -sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

H1299 model

Very High 12,5%

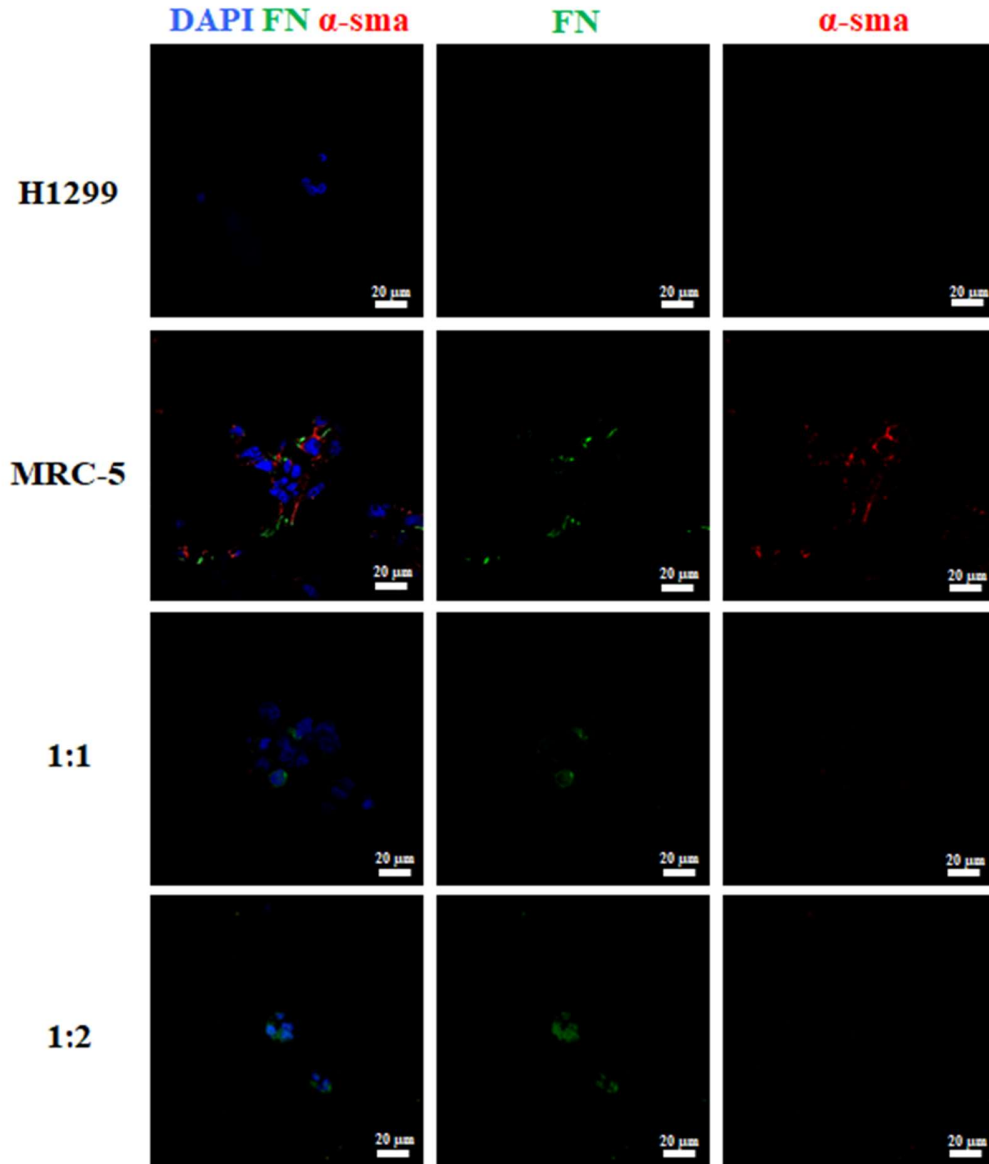


Fig. 2.19: Immunofluorescence analysis on H1299 model cultured in GelMA Very High 12.5%. The staining was performed after 14 days of culture. Fibronectin (FN) and α-sma were detected in FITC (green; Exc = 488 nm, Emm = 475/28 nm) and TRITC (red, Exc = 561 nm Emm = 575/25 nm) channels, respectively. Nuclei were stained by DAPI (blue, Exc = 405 nm, Emm = 390/22 nm).

2.3.9 Analysis of the expression of genes involved in ECM production and modification

After the analysis of the protein levels, the corresponding gene expression was investigated. As shown in Fig. 2.20-23 several genes were analyzed: α -SMA, Col1A1, Fibronectin, metalloproteinases type II (MMP2) and TGF- β . As expected, α SMA expression was greatly enhanced after 14 days of culture in MRC-5 in the mono-culture, confirming the previous analysis. Instead, Col1A1 and Fibronectin genes expression were mainly decreased after 14 days. This result was quite unexpected, because of the outcomes of protein studies. Hence, we supposed that at this time point (14 days), the increase of proteins amount was linked to post-transcriptional modification rather than to modification in gene expression.

Consequently, we decided to analyze other molecules involved in cancer progression and ECM modification. The first one was the matrix metalloproteinase MMP2. This protein coding gene, is a Zn^{2+} -dependent enzyme, which is able to digest several components of the ECM, including various collagens and glycoproteins (e.g. fibronectin)³⁸. Further, its expression is strongly increased in lung cancer than in healthy tissue and it is associated with poor prognosis³⁴. Surprisingly, as shown in Fig. 2.20-23, MMP2 was highly expressed in MRC-5 after 1 day of culture, but this modulation was lost after 14 days of culture. Moreover, its expression was statistically significant lower compared to the mono-culture, confirming again a strong influence of the tumor cells towards the behavior of the fibroblast.

Another player in the cross-talk between tumor cells and the tumor microenvironment is TGF β , which is a cytokine involved in the activation of several intracellular transducers (e.g. Smad, MAP kinases, PI3K/AKT pathways)³⁹. In lung cancer, TGF β has an initial suppressor role for its capacity to reduce cell proliferation, whereas it acquires an opposite behavior in cancer progression, during which it promotes epithelial-to-mesenchymal transition and facilitates invasion and metastasis through the modulation of the tumor microenvironment⁴⁰. As expected, its expression was detectable in both tumor cells and MRC-5. Interestingly, its behavior in the co-culture depended on matrix physical and mechanical proprieties. Indeed, its expression was significantly increased after 14 days of culture in Very High 10% for A549 model and in High 12,5% for H1299 model (Fig. 2.21 and 2.22). Obviously, we could not distinguish the contribution of the two cell types and further analyses should be performed to dissect the role of tumor cells and fibroblasts in the modulation of this cytokine. However, it was quite clear that also the matrix's properties played a role in the control of gene expression.

As shown, MRC-5 showed different behavior in the mono-culture compared to the co-culture with A549 or H1299, hence their behavior was further analyzed to understand the contribution of the matrix on gene expression. Variations in expression between day 1 and day 14 in different matrices were analyzed. Surprisingly, as presented in Fig. 2.24, α -SMA and Col1A1 expressions showed significant modulations. Indeed, α -SMA increase was greatly enhanced in High 10% and Very High 12,5%, whereas Col1A1 presented a strong increase only when MRC-5 cells were cultured into Very High 10%. This analysis suggested again that the matrix had an active role in the modulation of these genes, even if further analysis were required to confirm this crosstalk.

A549 model

High 10%

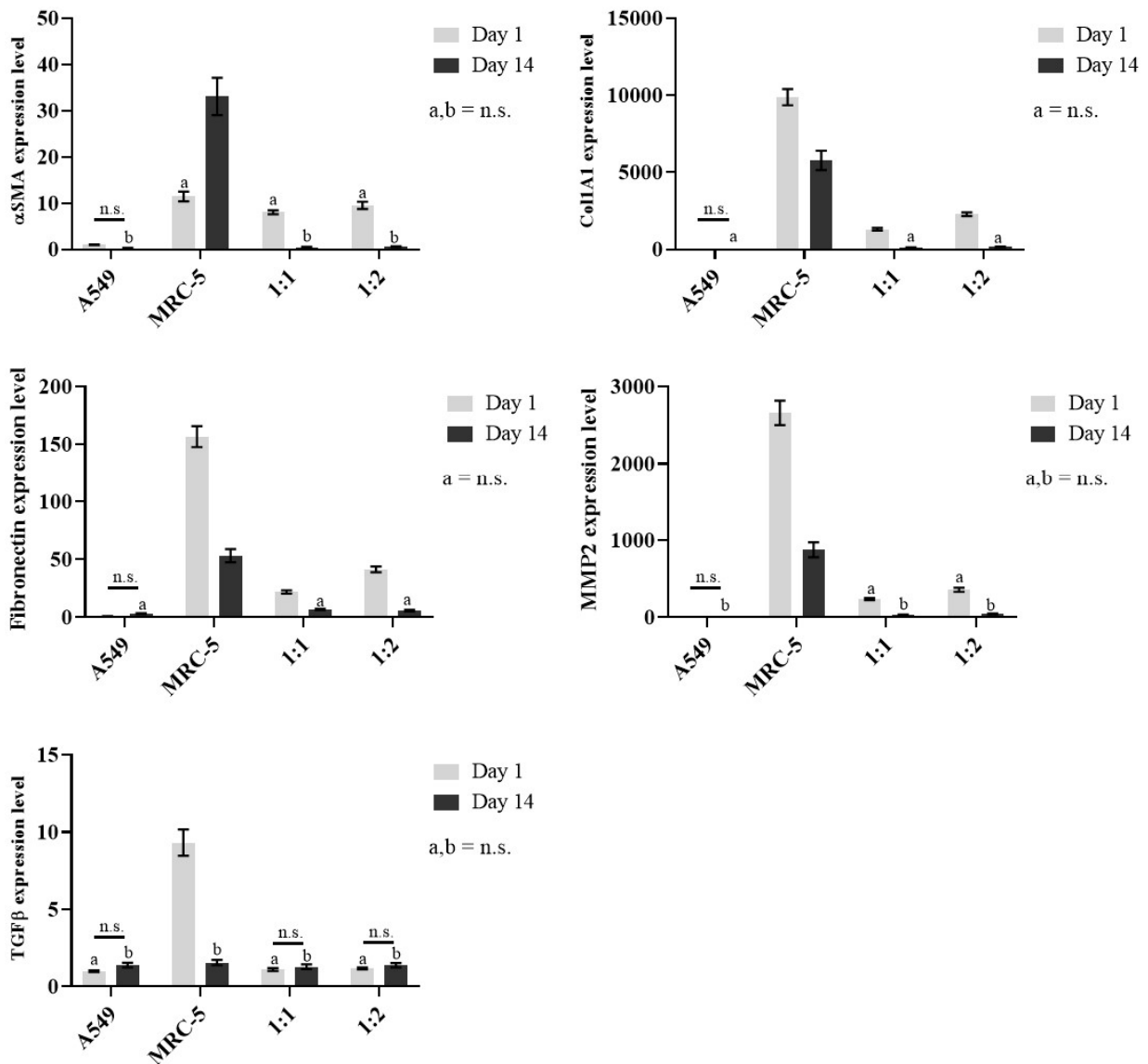


Fig. 2.20: qRT-PCR analysis of A549 model grown in High 10%. RNA expression was analyzed after 1 day and 14 days of culture. Results were shown as $\Delta\Delta C_t \pm SD$. Only not statistically significant differences were indicated.

A549 model Very High 10%

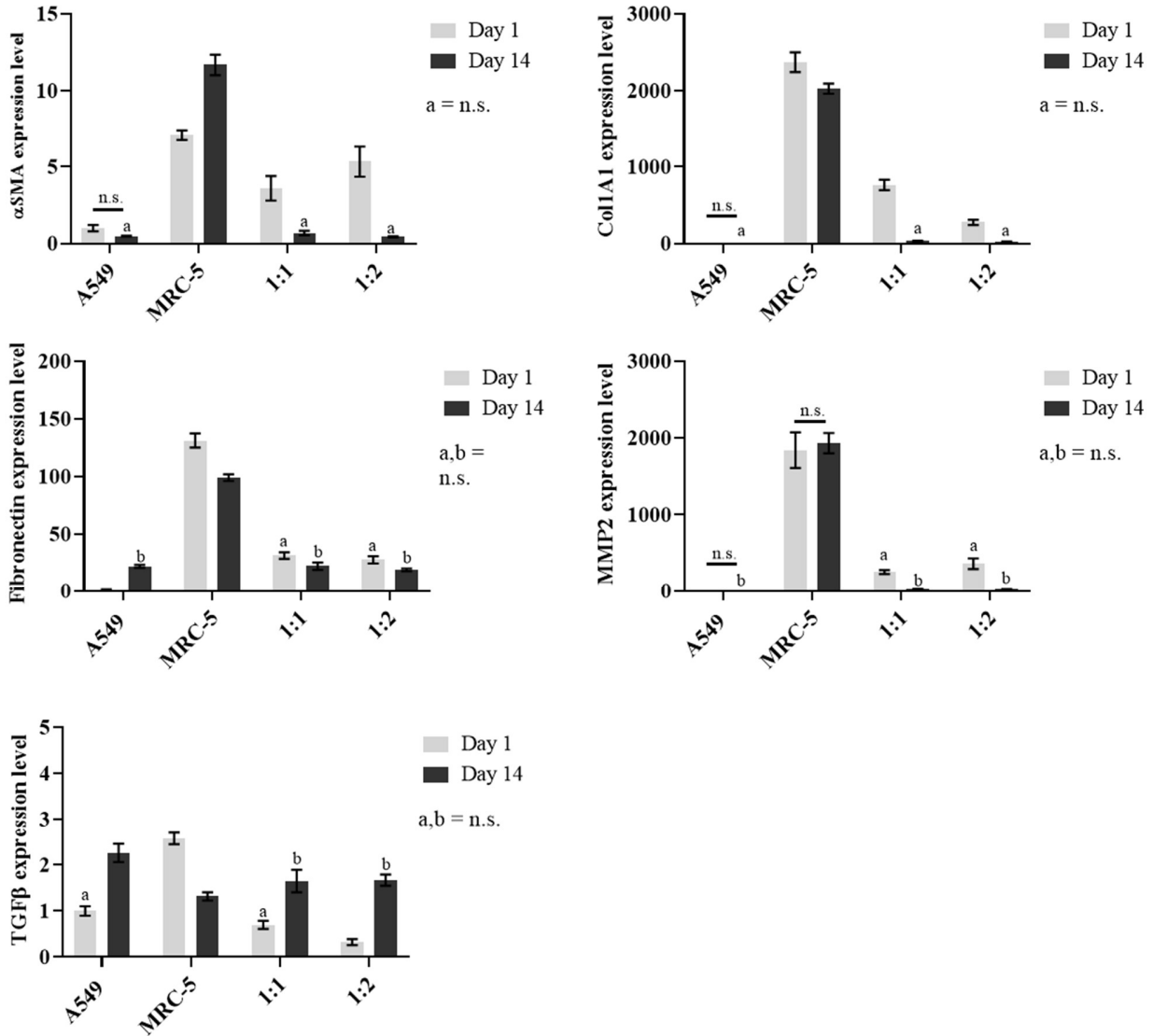


Fig. 2.21: qRT-PCR analysis of A549 model grown in Very High 10%. RNA expression was analyzed after 1 day and 14 days of culture. Results were shown as $\Delta\Delta Ct \pm SD$. Only not statistically significant differences were indicated.

H1299 model

High 12,5%

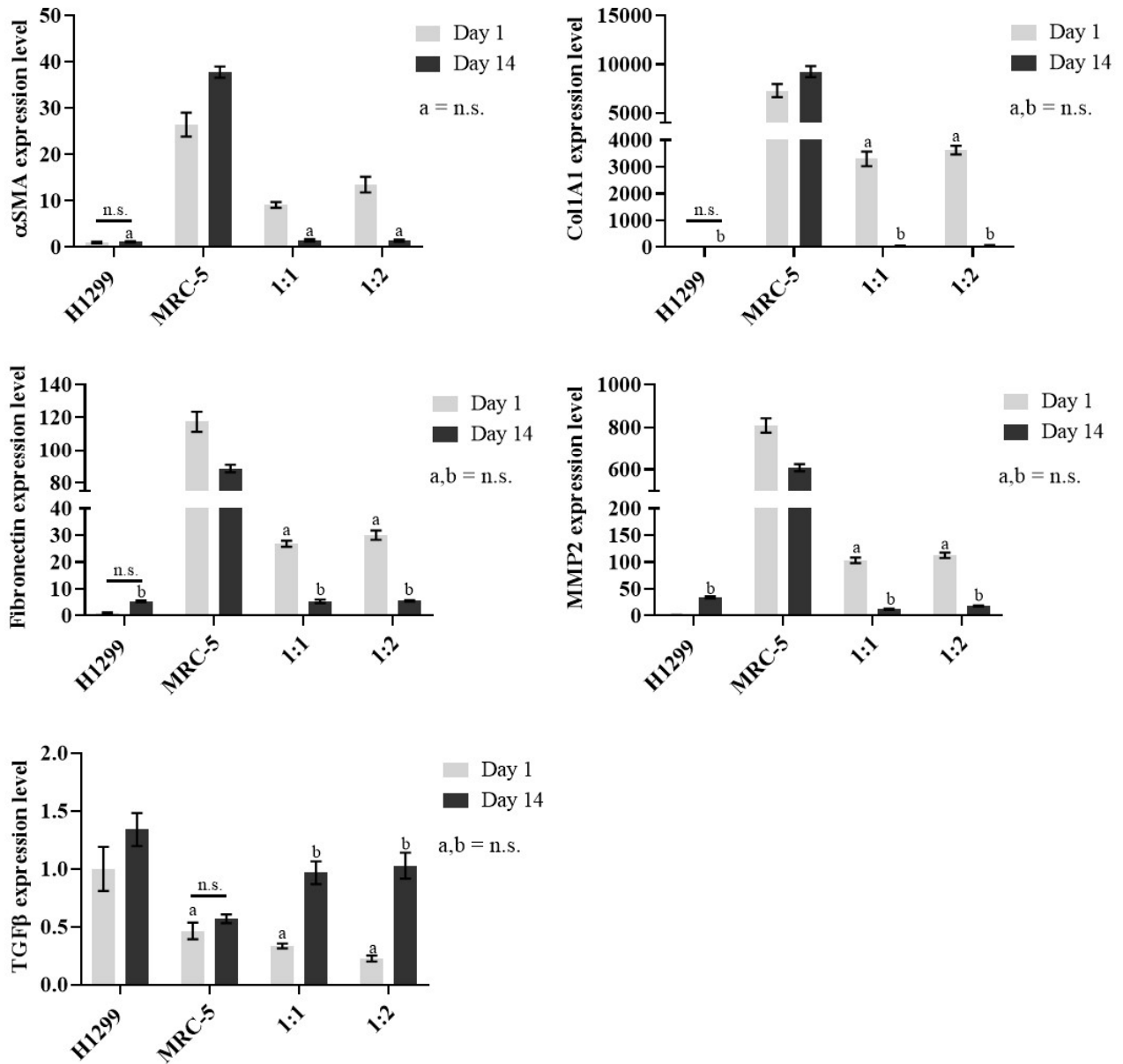


Fig. 2.22: qRT-PCR analysis of H1299 model grown in High 12.5%. RNA expression was analyzed after 1 day and 14 days of culture. Results were shown as $\Delta\Delta C_t \pm SD$. Only not statistically significant differences were indicated.

H1299 model Very High 12,5%

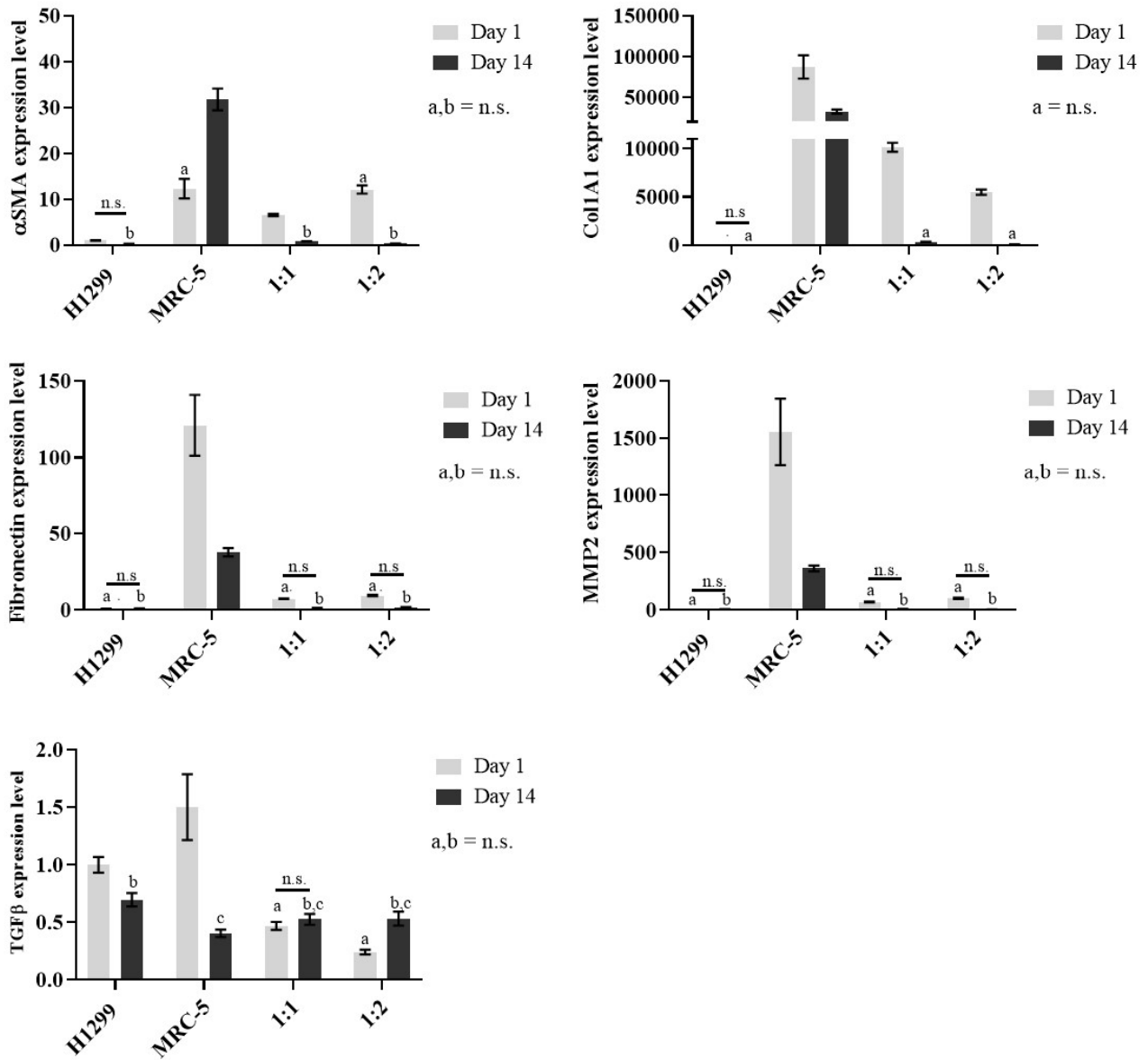


Fig. 2.23: qRT-PCR analysis of H1299 model grown in Very High 12.5%. RNA expression was analyzed after 1 day and 14 days of culture. Results were shown as $\Delta\Delta Ct \pm SD$. Only not statistically significant differences were indicated.

MRC-5

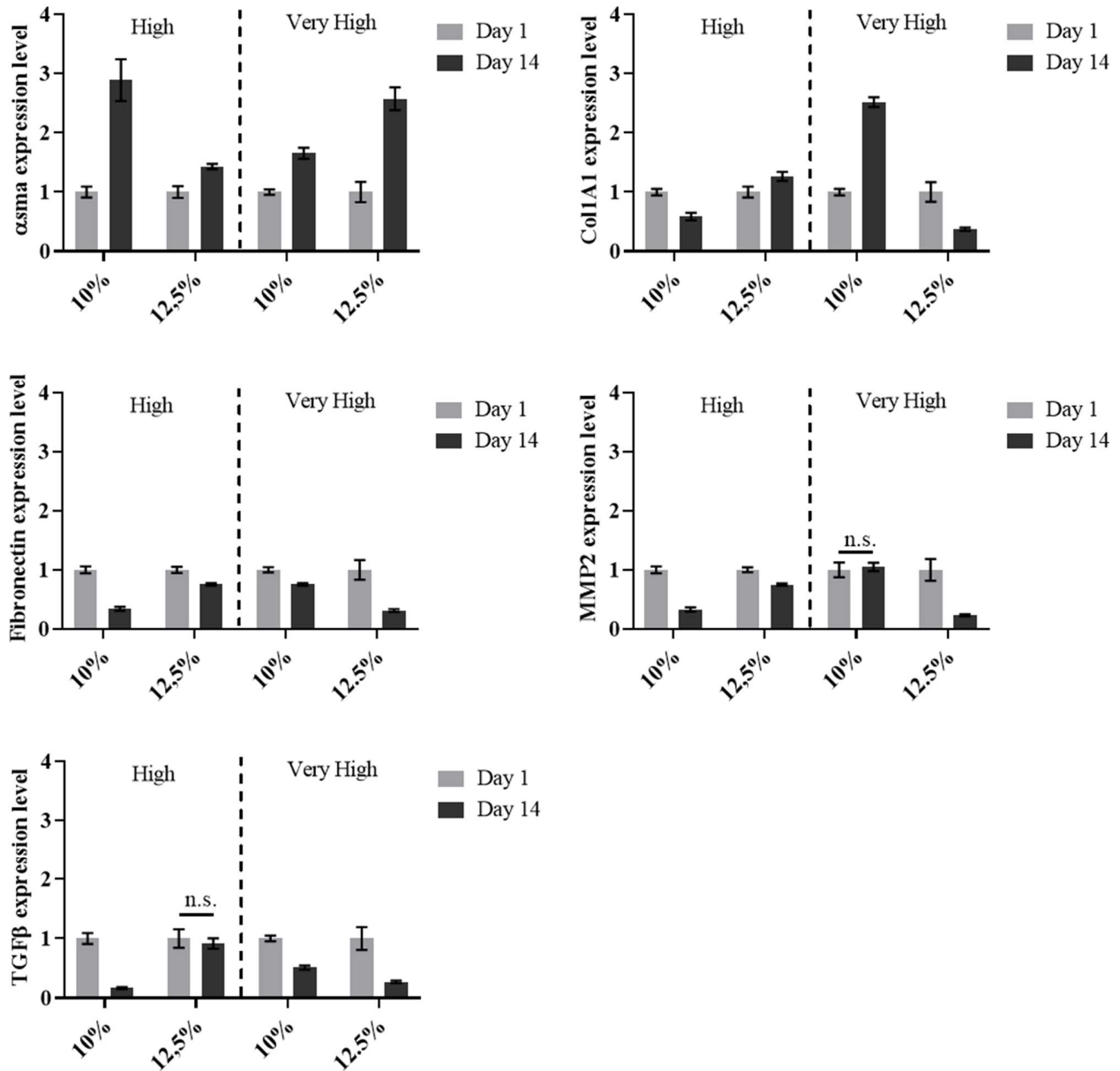


Fig. 2.24: qRT-PCR analysis of MRC-5 grown in different matrices. RNA expression was analyzed after 1 day and 14 days of culture. Results were shown as $\Delta\Delta Ct \pm SD$. Only not statistically significant differences were indicated.

2.4. Conclusions

This study demonstrated the feasibility of using GelMA formulations for the establishment of long-term 3D cell culture. Specifically, GelMA at different degrees of substitution was synthesized, by modulating the amount of methacrylic anhydride during the reaction. LAP was selected as photoinitiator, because of its water solubility, biocompatibility and absorbance in the visible range. Importantly, DoFs modulation and variation in the concentration of gelatin in the photopolymerized hydrogel had a strong impact on its physical properties, specifically, in terms of swelling and elastic modulus. These results demonstrated the need to deeply investigate cell behavior in the 3D culture, to detect any differences linked to different GelMA formulations. Further, since the goal of this study was mimicking tumor niche features, co-culture of tumor cells and fibroblasts were analyzed as well. Two lung adenocarcinoma cell lines, A549 or H1299 cells, which carried distinct mutational signatures, were cultured with normal lung fibroblasts (MRC-5) at two different ratios, i.e. 1:1 and 1:2. The initial screening was done by assessing the proliferation rate of both the co-cultures and the results led to the selection of two different GelMA matrix for each model, in particular, GelMA High and Very High, at 10 w/v% for A549 and GelMA High and Very High at 12,5 w/v% for H1299 model. Importantly, the co-culture models were also maintained over 14 days of culture without noticing decreasing in cell viability. Molecular analysis showed a strong activation of normal fibroblasts, when cultured in a 3D environment, indeed, α -sma, collagen type I, fibronectin and MMP2 expression was greatly enhanced, but this upregulation was completely lost in the co-culture, suggesting a cross-talk between the two populations that induced changes in MRC-5 behavior. Further, the cytokine TGF β gene expression was increased in the co-culture, after 14 days of culture. All these modulations were not the same in the different GelMA models analyzed, suggesting an active role of the matrix in gene expression activation or repression. In the next future, the effect of the tumor cells on fibroblast will be analyzed, knowing that senescence insurgence or the establishment of an inflammatory microenvironment, could cause changes in MRC-5 behavior. Further, to better characterize cell response to the mechanical signal, transcription factors and intracellular pathways linked to mechanotransduction will be investigated.

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Chapter 3

Lung adenocarcinoma spheroid formation in 3D bioprinted GelMA

Abstract

In this chapter, the previously described GelMA formulations were used as a supporting matrix for the formation of spheroids starting from lung adenocarcinoma cell line A549. Specifically, the aim of the work was the 3D printing of a reliable platform for the study of lung cancer constructs evolution, indeed, the optimization of printing parameters and designing of precise and effective shapes could increase the soundness of the model. In particular, cell density, GelMA physical properties and 3D printed geometry were modulated to increase the quality of obtained aggregates and help the formation of optimal spheroids, too.

3.1. Introduction

Over the years, 3D culture has been acquired huge importance in the studies of various diseases and in the development of effective drug treatments ¹⁻³. Importantly, the main used platforms were spheroids ⁴ and organoid⁵ and, since lung cancers still represent the main cause of cancer-related death ⁶, optimized protocols for its growth has been obtained ^{7,8}. Specifically, lung cancer spheroids could be derived by using scaffold-free methods like hanging drop, magnetic levitation and rotary cell cultures approaches ⁹. However, since the importance of the ECM in the tumor niche during disease progression ¹⁰, spheroids could be grown also in several matrices among which Matrigel was the most used one ^{11,12}. Unfortunately, basement membrane extracts presented two main drawbacks, high batch-to-batch heterogeneity, which decreased experimental reproducibility and the need of a temperature-controlled environment to avoid early polymerization ¹³. The use of GelMA formulations, described in the previous chapters, could overcome these limitations. Indeed, the feasibility of reproducible results regardless of the synthesized GelMA batches and the controlled photopolymerization have been demonstrated. Further, all GelMA formulations supported lung cancer cell viability during long term culture. In this study, the versatility of GelMA to develop *in vitro* platform for NSCLC spheroid formation and growth was exploited. In this preliminary analysis, A549 density, GelMA formulations and 3D printing parameters were optimized to obtain a reliable scaffold. Since this study represents a proof-of-concept of the feasibility to use printed GelMA as substitution of traditional matrix, only A549 cell line was initially used.

3.2. Materials and Methods

3.2.1. Materials

For GelMA synthesis and cell culture maintenance, the reagents described in Chapter 2 have been used. For proliferation assay, Resazurin has been bought from Sigma-Aldrich (R7017), whereas WGA Alexa Fluor® 647 conjugate was obtained from Thermo-Fisher (W32466).

3.2.2. *In vitro* cell culture

All experiments were performed using lung cancer cell lines A549 cultured in BenchStable RPMI 1640 medium with GlutaMAX adding 10% of FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

3.2.3. Spheroid formation

To test spheroids formation in a 96-well plate, they were coated with 30 µl of the selected GelMA. After 2 minutes of incubation at 37°C, 96-well plates were irradiated with a LED at a wavelength of $\lambda = 405$ nm for 1 min to induce polymerization. After coating formation, A549 cells were suspended at different cell densities (3×10^3 , 1×10^4 and $1,5 \times 10^4$ cells/50 µl) in the 37°C pre-heated GelMA formulations and seeded onto the 96-well plate (50 µl/well). Immediately after seeding, the plates were further irradiated for 2 min. Finally, 200 µl of cell culture medium were added to each well and replaced every 5 days, until the end of the experiment.

Spheroid images were captured after 12 days of culture with Eclipse Ti2 Nikon (Tokyo, Japan) microscope equipped with a Crest X-Light spinning disk.

For 3D printing experiment, cell-laden GelMA at the density of $1,5 \times 10^4$ cells/50 µl was extruded using a 3 mL UV-secure pneumatic-driven extrusion printhead and a nozzle with an inner diameter of 250 µm, into 6 well suspension plates. The printing speed was set at 15 mm/s and the pressure was approximately at 0,080 MPa. G-codes were generated using BioCAD software (REGENHU, Switzerland). Three different architectures were generated: round, ring and grid, with the same amount of cell-laden GelMA. Each geometry consisted of 4 layers, each one with a thickness of 500 µm, for a final thickness of 2 mm. After printing, samples were photopolymerized by using a UV oven (wavelength 365 nm with a power of 10 mW/cm²) and submerged in 5 mL complete cell culture medium which was replaced every 5 days until the end of the experiments.

3.2.3. *In vitro* metabolic assay

To test cell viability, 3D bioprinted samples were analyzed after 6 and 12 days of culture, Specifically, 3D constructs were transferred in a new well plate and 500 µL of Resazurin at the concentration of 0,1 mg/mL was added to each well. After 4 hours of incubation at 37°C, the solution was transferred in a white 96-well plate (200

$\mu\text{L}/\text{well}$) and the fluorescence signal was detected by using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA). Empty printed structures were used as negative control.

3.2.4. *In vitro* imaging analysis

For imaging analysis, 3D bioprinted samples were washed with DPBS and fixed with 4% of paraformaldehyde for 1 hour at RT. Then, samples were washed again three times with DPBS and stored at 4°C until staining. For membrane staining, fixed samples were incubated with 5 $\mu\text{g}/\text{mL}$ WGA Alexa Fluor® 647 conjugate (Exc = 640 nm, Emm = 637/12) for 1 hour at RT. Then, they were permeabilized with 0,25% Triton x-100 for 30 min and stained with 0,4 mM DAPI (Exc = 405 nm, Emm = 390/22 nm) for 3 hours, always at RT. Fluorescent images were collected using an Eclipse Ti2 Nikon (Tokyo, Japan) microscope equipped with a Crest X-Light spinning disk.

For the analysis of spheroid diameter, at least five images for each architecture (round, ring, or grid), for GelMA formulation selected (7,5% High and Very High) and time point (6 and 12 days) were analyzed manually, by using the software ImageJ. The diameters were obtained arithmetically, and their distribution was expressed in terms of frequency (%) of each diameter in each geometry.

3.3. Results

3.3.1. Optimization of cell density for spheroids formation in GelMA

Initial cell density played a crucial role in obtaining effective tumor spheroids⁴, hence, it was the first parameter evaluated. Specifically, a systematic analysis was conducted by assessing bright field microscopy images of each cell concentration on A549 spheroid formation and growth. Indeed, optimal spheroids were identified as cell aggregates with a uniform morphology, round shape and defined external boundaries. In addition, they should appear as a translucent ball, with a darker core and increasing dimensions¹⁴. To select the optimal seeding density, GelMA Medium at the concentration of 10 w/v% was initially used as a scaffold for spheroid formation. Indeed, from previous analysis, A549 mono-culture showed good cell viability in all GelMAs. Initially, a low amount of A549 (i.e. 1×10^3 , 2×10^3 , 4×10^3 and 8×10^3 cells/ $50 \mu\text{L}$) was selected (Fig. 3.1). As expected, a clear correlation between initial cell number and spheroid formation after 12 days of culture could be observed. However, we excluded the lowest densities, i.e., 1×10^3 and 2×10^3 cells/ $50 \mu\text{L}$, because of the absence of spheroids formation (Fig. 3.1).

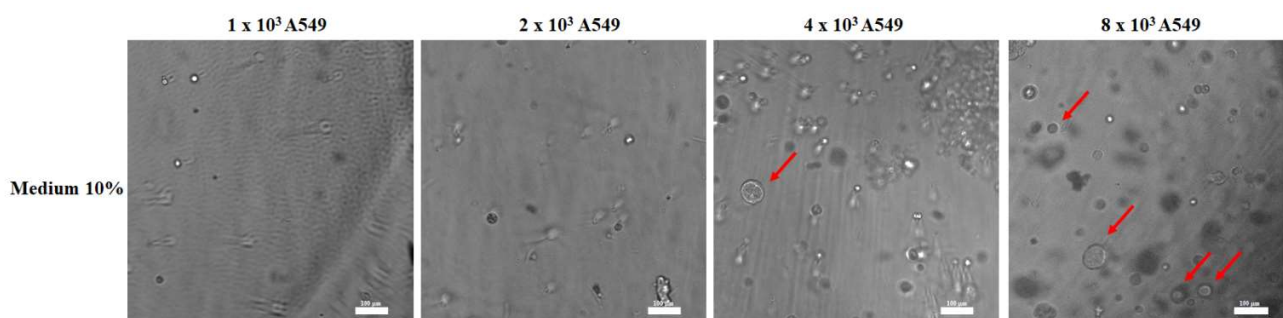


Fig. 3.1: Bright field images of A549 cells seeded at different densities (i.e. 1x10³, 2x10³, 4x10³ and 8x10³ cells/50µL) and cultured in GelMA Medium 10% formulation for 12 days. Scale bar: 100µm.

Hence, higher concentrations were used (Fig. 3.2). A distinct difference was visible between 5x10³ and 1x10⁴ cell seeding densities, with a higher number of cell aggregates detected in the second one. However, increasing the densities to 1,5x10⁴ cells/50µL did not strongly improve spheroid formation, suggesting that higher initial cell concentrations were not required for effective aggregates induction (Fig. 3.2). Since the higher homogeneity and clear spheroids formation, cell density of 1.5x10⁴ cells/50 µL was selected for further analysis.

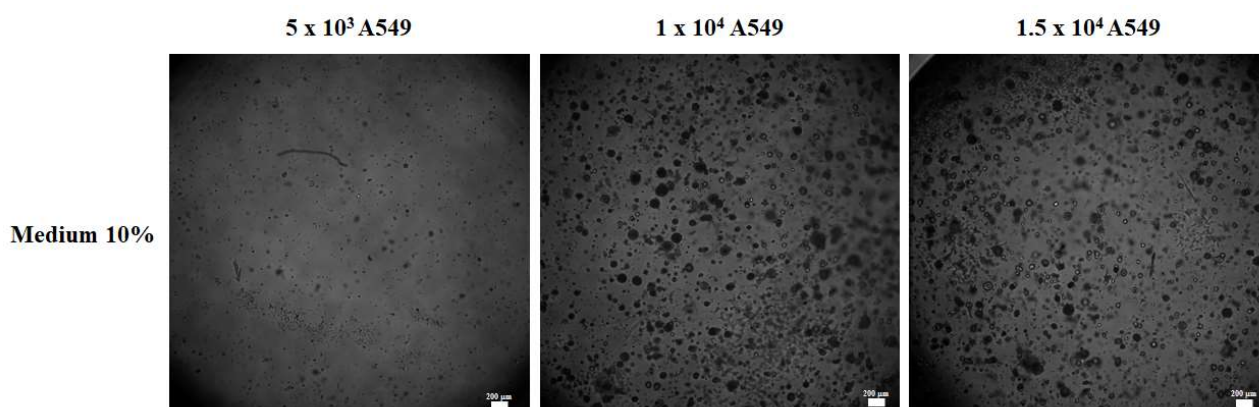


Fig. 3.2: Bright field images of A549 cells seeded at different densities (i.e., 5x10³, 1x10⁴ and 1.5x10³ cells/50µL) and cultured in GelMA Medium 10% formulation for 12 days. Scale bar: 200 µm.

3.3.2. Selection of optimal GelMA formulation

The second question that had to be addressed was the types of GelMA suitable for optimal induction of spheroid formation. Indeed, it has been shown, that the mechanical properties of the scaffold could have an impact on cell behavior and drug response¹⁵. In the analysis described in Chapter 2, Low formulation has been excluded, because of cell leakage due to the softness of the matrix. As already shown in the previous analysis, almost all the cells reached the bottom of the plate and started to grow in a 2D way (Fig. 3.3). Better results were obtained by increasing the concentration to 10 w/v%, but the quality of the aggregates remained low and cell sedimentation was still present (Fig. 3.3). Hence, also in this case, the lower DoF was excluded from further investigations.

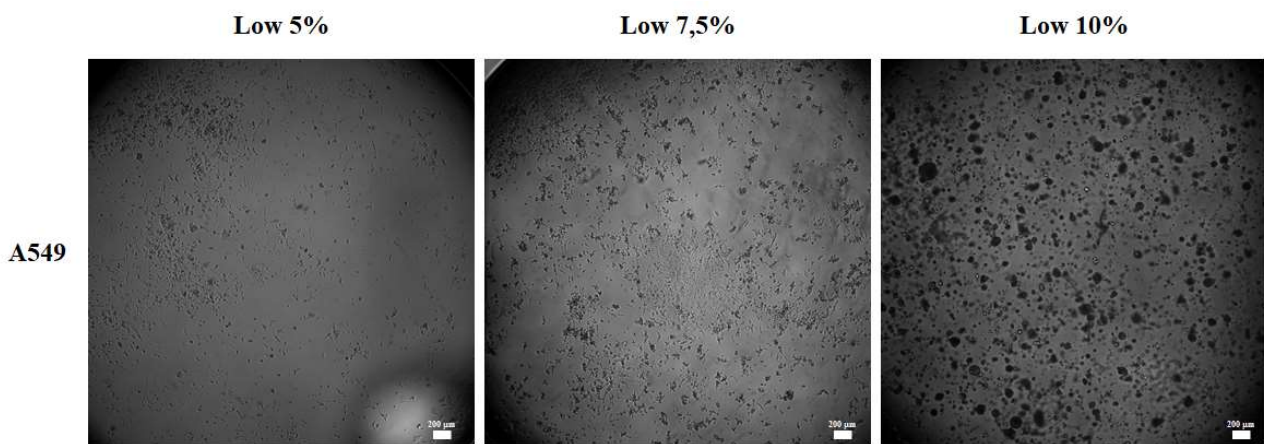


Fig. 3.3: Bright field images of A549 cells seeded in GelMA Low at different concentration (i.e. 5; 7,5 and 10 w/v%) and cultured for 12 days. Scale bar: 200 µm.

At this point, the results obtained by using GelMA Medium, High and very High at two different concentrations (i.e. 7,5 and 10 w/v%) were assessed. Representative bright field images were acquired after 12 days of culture and were shown in Fig. 3.4. Interestingly, High and Very High formulations at 7,5 w/v% showed higher amounts of spheroids with a round cell morphology compared to Medium GelMA, in which there were many single cells and smaller aggregates. On the contrary, in 10 w/v % GelMA Medium appeared to be the best condition, even if not comparable with High and Very High at 7,5 w/v%. Indeed, in GelMA High and Very High formulation at 10 w/v% fewer round shape spheroids were detected and most of cells did not aggregate in compact spheres (Fig. 3.4). Thanks to these preliminary investigations, GelMA High and Very High 7,5 w/v% were chosen as matrix bioinks for spheroid formation in 3D bioprinted structures.

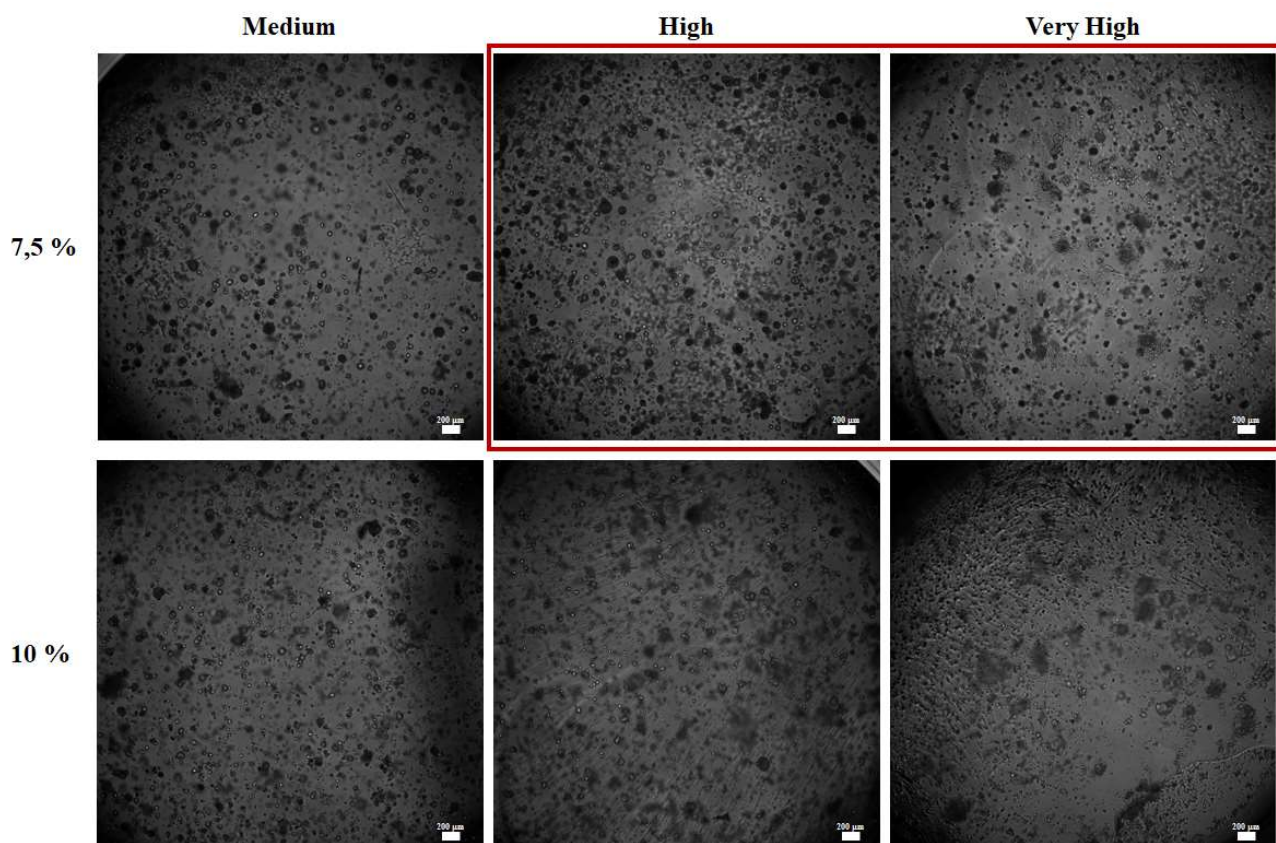


Fig. 3.4: Representative bright field images of A549 cells seeded in GelMA Medium, High and Very High at two different concentrations (i.e. 7,5 and 10 w/v%) and cultured for 12 days. Scale bar: 200 μm .

3.3.3. Optimization of 3D printing parameter and evaluation of spheroid formation

To investigate the potential of GelMA bioinks to produce printed 3D models for the study of cancer spheroids, three different architectures were designed and translated in G-code using BioCAD software (regenHu, Switzerland): a round, a ring and a grid (Fig. 3.5a). Structures were printed using the same amount of cell-laden GelMA and consisted of 4 layers, each one with a thickness of 500 μm . To obtain objects with high printing fidelity, the pressure was set at 0,080 MPa and the speed at 15 mm/s. Since cell aggregation in bioinks represented a problem for extrusion-based bioprinting¹⁶, the formulations were kept in ice for 10 minutes and the printing temperature was set at 12°C to avoid this drawback. Further, we also evaluate the ratio between surface area and volume of the structures to understand where cells were more easily reached by the nutrients and oxygen. The following values were found: 12,8, 21,3 and 49,0 for round, ring and grid architecture, respectively. As expected, the latter showed the best ratio.

Importantly, to reduce the time of photopolymerization which led to loss of resolution, constructs were irradiated with an UV oven at 365 nm. Then, the proliferation rate of spheroids cultured in printed structure was analyzed. As presented in Fig. 3.5b,c, all the conditions showed an increase in cell proliferation rate between day 6 and day 12 indicating the feasibility of using printing for obtaining effective models. Interestingly, as expected from the results of the ratio between surface area and volume, grid conformation was the one with the higher enhancement in cell proliferation demonstrating that when spheroids were more exposed to nutrients and oxygen, they grew faster. Further, grid structure printed with Very High formulation showed better results compared to all the other conditions (Fig. 3.5c).

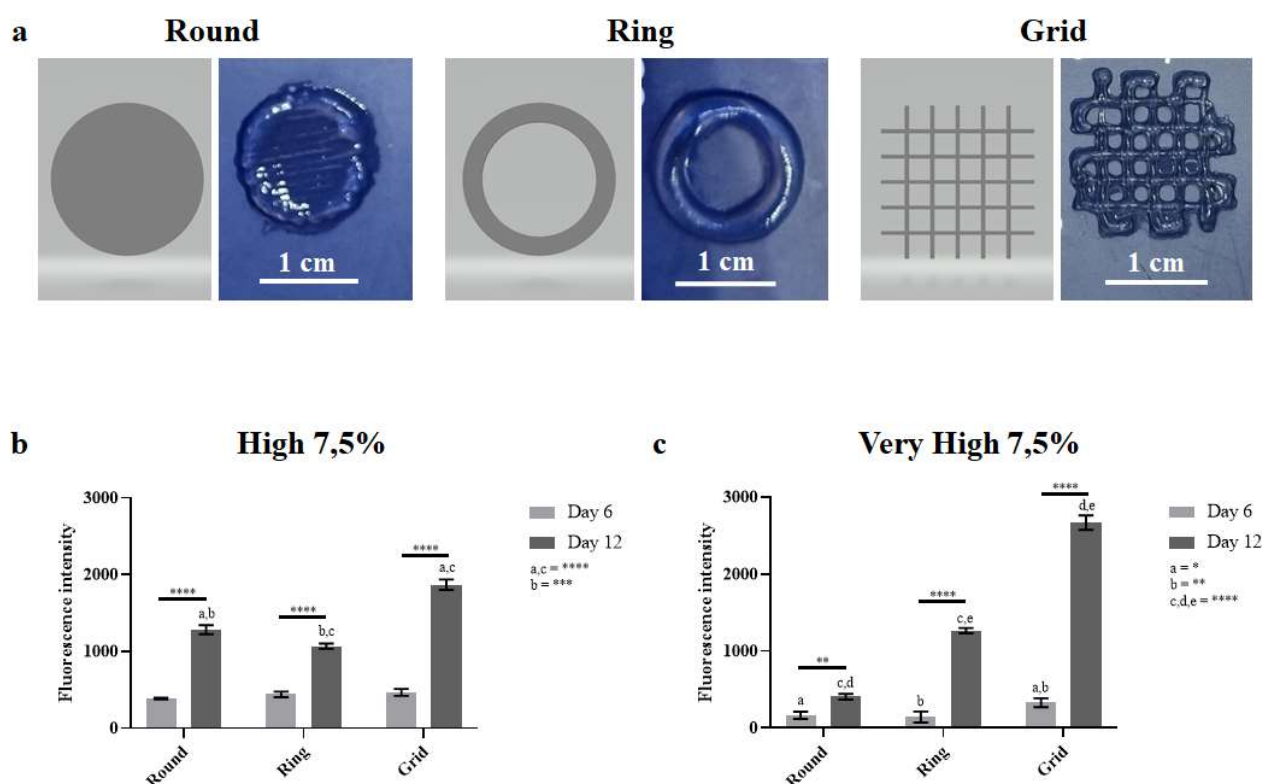


Fig. 3.5: a) STL images of a horizontal sections of printed structure and representative images of those. b,c) Resazurin analysis of proliferation rate of printed spheroids in GelMA High 7,5 v/w% (b) and Very High 7,5% (c) after 6 and 12 days of culture. Statistical analysis was performed with two-ways ANOVA (* p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).

To elucidate the properties of obtained spheroids, they were analyzed also by using confocal fluorescence microscopy, after 6 and 12 days of culture. Samples were fixed at the selected time points and stained with wheat germ agglutinin (WGA) Alexa Fluor 647 conjugated, which bound to sialic acid and N-acetylglucosaminyl residues and resulted in cell surface staining allowing the measurement of cells and aggregates diameters (Fig. 3.6).

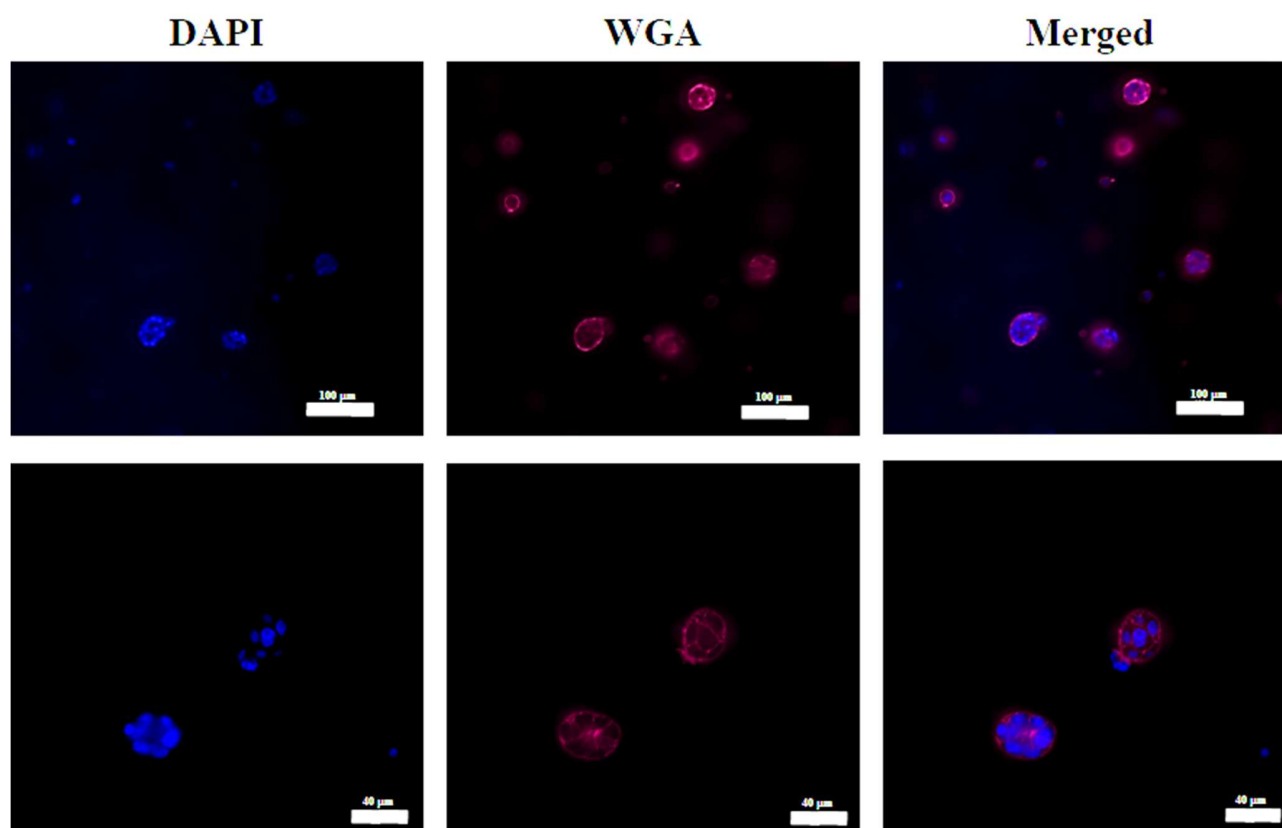


Fig. 3.6: Representative images of spheroids grown in GelMA High 7,5 w/v% stained with WGA and DAPI. Scale Bar: 100 µm and 40 µm in the upper or lower line of the figure, respectively.

The frequencies of aggregates of different diameters were analyzed (Fig. 3.7). As expected, an increase in size was observed in all samples over time, with similar trends in both High and Very High formulations. However, grid architecture supported the growth of aggregates with higher diameters. In detail, 10% of the aggregates in GelMA High 7,5 w/v % and 4% of the aggregates in GelMA Very High 7,5 w/v % exceeded 100 µm diameter after 12 days. An increasing trend was also observed for ring architecture in both High and Very High formulations, which displayed frequency class with higher diameters compared to the round one (Fig. 3.7). This increase in spheroid growth could be ascribable to the better exchange of oxygen and nutrients in these kinds of architecture, due to the higher surface area exposed to the cell culture medium (grid > ring > round). This test shows as 3D bioprinting and the implementation of different geometries could be crucial to improve the effectiveness of *in vitro* models. Both High 7,5 w/v % and Very High 7,5 w/v % GelMA formulations could be optimal bioinks, where explore spheroid formation and growth.

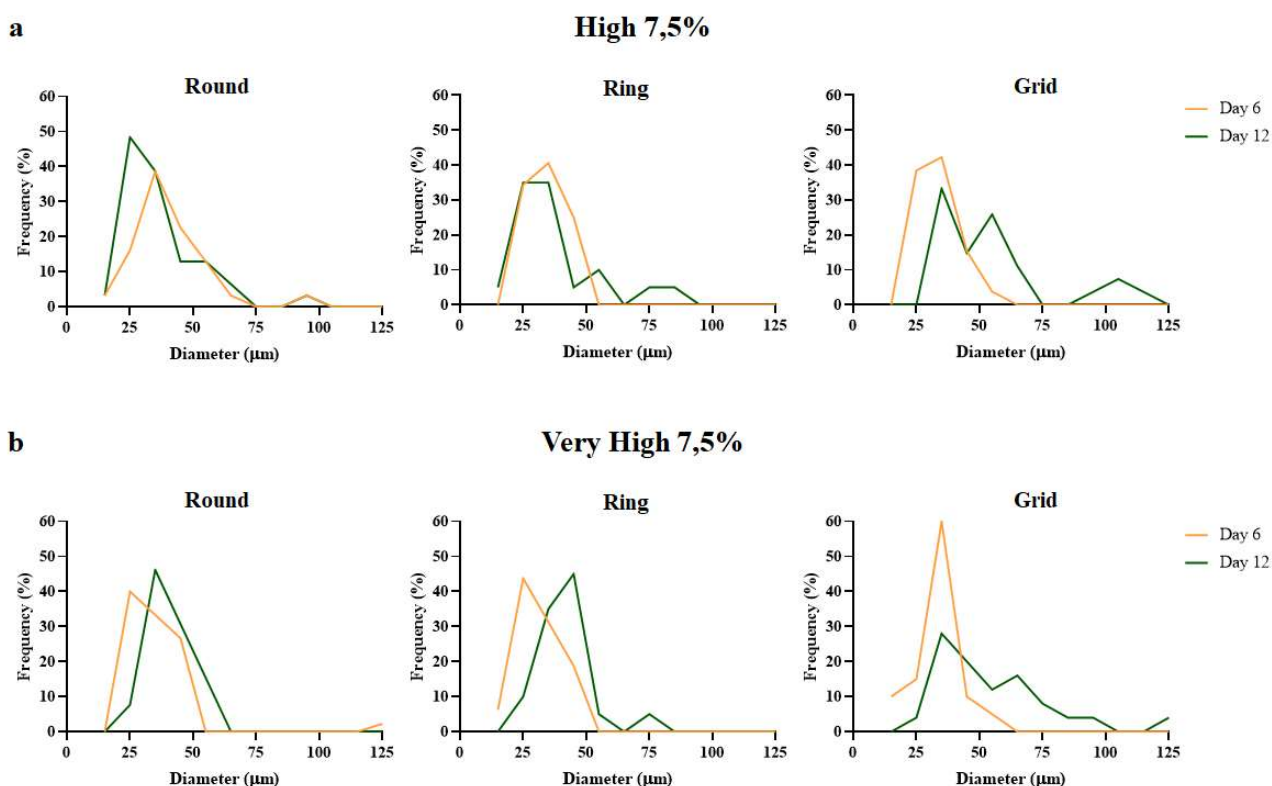


Fig. 3.7: Analysis of aggregates diameters measured after 6 and 12 days of culture expressed as frequency of each diameters class for GelMA High 7,5 w/v% (a) and Very High 7,5 w/v% (b) biprinted architecture.

3.4. Conclusions

The results described in this chapter showed once more the versatility of GelMA as a supporting matrix for cell culture. A549 spheroids were successfully obtained in two different GelMA formulations (i.e. High and Very High at 7,5 w/v%). Moreover, printing parameters have been optimized to reduce cell death and to avoid impairment of the capability of aggregate formations. Further, the importance of efficient geometries scaffold design to support nutrients and oxygen delivery has been demonstrated. This analysis showed how extrusion-based bioprinting could be used to obtain a versatile platform for lung cancer studies. In the next analysis, the capability of other cell types (e.g. H1299 cells described in the previous chapter) to produce viable spheroids in GelMA matrix should be tested. Further, as already described in chapter 3, GelMA could supported also co-culture, thus future experiments will be conducted to form multi-cellular spheroids (i.e., tumor cells and fibroblasts) to investigate the interaction which occurred between different cell types. At this point, the optimized models could represent a valuable platform for

drug screening analysis to better predict the response of NSCLCs to new molecules to select the most promising ones before the analyses.

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Chapter 4

Characterization and printing of Carbopol-based bioinks

Abstract

This chapter describes the characterization and the printing optimization of Carbopol-based bioinks. The aim of this study was the optimization of a low-cost and easy-to-obtain material, which does not require an additional step after the printing process, as the application of light for photopolymerization. To achieve that, three different Carbopol formulations (EDT 2020 NF, Ultrez 10 NF and NF-980) were tested, in terms of printability and biocompatibility. Rheological analysis demonstrated the feasibility of using these bioinks for 3D printing, thus, tests were conducted by using an extrusion-based bio-printer (BIO-X, Cell Link). Promising results in terms of printing resolution were obtained for EDT 2020 NF and Ultrez 10 NF formulations. Since the idea is to use them in a lung cancer model, their biocompatibility and cell toxicity were analyzed on lung cancer epithelial cells (A549) and normal lung fibroblasts (MRC-5). The obtained outcomes showed that EDT 2020 NF formulation was a promising candidate for 3D printing, both in terms of printing performance and cell viability. The results presented in this chapter have been already published in Baruffaldi et al., 2021 ¹.

4.1. Introduction

3D bioprinting represented a promising strategy to obtain complex structures that mimicked tissue organization. Over the years, many approaches were developed, specifically, the most used techniques were inkjet, laser-assisted or micro-extrusion-based bioprinting ². The latter presented several advantages: first, it was compatible with a wide range of materials. Nonetheless, the resolution was lower compared to inkjet printing and several parameters including needle dimension and printing pressure needed to be optimized ³. However, the flexibility and relative cheapness strongly influence the choice of this approach for bioprinting. Indeed, this technique was used for obtaining scaffolds, which included inside a microfluidic structure that mimicked the physiological vasculature. Unfortunately, the shear stress applied to cells during the printing process could strongly impact on cell viability and functional tissue model development. Importantly, this limitation could be overcome by using bioinks characterized by shear thinning behavior ⁴. Further, cell viability after printing was strongly correlated to bioinks' viscosity, nozzle size, tip clogging and cell concentration ⁵. For these reasons, inks development played an important role in the effectiveness of tissue model printing. A systematic review found Alginate and PCL to be the most used ink ⁶ as well as photo-crosslinkable ones which were widely used for extrusion based printing. Indeed, methacryloyl groups were usually added to gelatin ⁷ or hyaluronic acid ⁸ to improve their mechanical properties after hydrogel polymerization and printing resolution. Unfortunately, the use of photoinitiators and, of UV wavelength to initiate the reaction within the process of crosslinking could influence cell viability and behavior

^{9,10}. For this reason, the development of materials, which do not require these steps could improve biological outcomes. A promising candidate could be Carbopol, a soft granular microgel, which consisted of synthetic high-molecular weight polyacrylic acids which could be cross-linked with allyl sucrose or allyl pentaerythritol. It was widely used as a rheological modifier in pharmaceutical products. An important property of Carbopol was its capability to swell in an aqueous solution forming a gel after pH neutralization ¹¹. Thanks to its mucoadhesive properties, several types of Carbopol have been used in drug formulations ^{12–14}. For instance, Carbopol 934P was added to decrease the clearance, and so to increase the bioavailability, of drugs in intranasal formulations ^{15,16}. In previous studies, different types of Carbopol microgels have been already investigated, analyzing their printability and the possibility of using them as 3D scaffold for subsequent cell cultures, obtaining promising results ^{17,18}. In this chapter, Carbopol potential as bioink was further investigated. Specifically, three different Carbopol formulations (ETD 2020 NF, Ultrez 10 NF and NF-980) were evaluated by using an extrusion-based bioprinter (BIO X, CELLINK). Rheological properties and printing resolution were assessed to confirm the feasibility of using selected inks, then, biocompatibility and cell survival post printing were analyzed.

4.2. Materials and Methods

4.2.1. Materials

In this work, three different types of Carbopol were selected: ETD 2020 NF, Ultrez 10 NF and NF-980 (Lubrizol Corporation, Wickliffe, OH, USA). Printing was performed using BIOX pneumatic-driven extrusion bioprinter (CELLINK). Lung adenocarcinoma cell line, A549, and normal lung fibroblasts, MRC-5, were kindly provided by Valentina Monica from University of Turin and by Chiara Tonda Turo from Politecnico di Torino. RPMI 1640 medium and MEM medium were purchased from Sigma-Aldrich as well as the supplements: fetal bovine serum or FBS (F9665) and penicillin/streptomycin. For metabolic analysis, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (M2003) was obtained from Sigma-Aldrich. For microscopy detection, cells were stained with CYTO-ID® Red long-term cell tracer kit (ENZO life science).

4.2.2. Carbopol formulations preparation

Three types of Carbopol were dissolved into cell culture medium (RPMI 1640 or EMEM) under continuous stirring at determined concentration: ETD 2020 NF and NF-980 at 1,2 w/v% and Ultrez 10 NF at 1,5 w/v%. After complete powder dissolution, the solutions were neutralized by using NaOH 5M to obtain maximum swelling.

4.2.3. Rheological analysis

Rheological measurements were performed at 25°C by using an Anton Paar rheometer (Physica MCR 302). For the analysis, two aluminum plates with a diameter of 20 mm were used in the parallel-plate mode. The gap was constant at 0,3 mm. The oscillatory tests consisted in shear stress that ranged from 0,001Pa to 100 Pa by keeping constant the frequency at 1 Hz. For shear thinning assessment, the shear rate range was set up from 1 to 2000 s⁻¹. The thixotropic curve was derived by applying a shear rate of 1 s⁻¹ for 25 s, of 100 s⁻¹ for 50 s, and again 1 s⁻¹ until the end of the analysis. All experiments were performed at least three times for each formulation.

4.2.4. Printing test

Printability of Carbopol formulations was tested by using a pneumatic-driven extrusion bioprinter (BIOX, CELLINK). A sterile 3 mL cartridge and standard sterile 25G conical nozzle were used for all printing experiments. The material flow was controlled by pressure and the printing speed was set at 16 mm/s. For cell experiment, the samples were printed by using a droplet mode by extruding material for 5 s. Flow rate of ETD 2020 NF, Ultrez 10 NF and NF-980 at different printing pressures were determined by using 3 mL cartridge with 25G conical needle.

4.2.5. Cell culture maintenance

All experiments were performed using A549 and MRC-5. They were cultured in RPMI 1640 medium or MEM medium, respectively, by adding 10% of FBS, 2mM Glutamine and 1% penicillin/streptomycin. All the cell types were maintained at 37°C in a humidified atmosphere with 5% CO₂.

4.2.6. Cell proliferation analysis

MTT assay was used for the assessment of the proliferation rate of cells cultured in 2D or 3D. Specifically, for the adhesion experiment, 96-wells were coated with Carbopol formulations, then, the cells were seeded at the density of 1,5x10⁴ cells/well and MTT analysis was performed after 6 and 24 hours. For the assessment of cell viability after printing, A549 or MRC-5 were suspended within Carbopol at the density of 3x10⁶ cells/mL and samples were plated in 96-multiwell plates. Not-printed Carbopol was used as a positive control. After 24 or 48 hours of incubation, 84 µL of 1 mg/mL MTT dissolved in PBS was added and after 3 h of incubation at 37°C, 250 µL of MTT solvent (10% SDS, 0.01 M HCl in H₂O) was used to solubilize the formazan crystals. Absorbance (i.e. 570 and 650 nm) was read after 1 h by using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA). Empty-Carbopol was used as a negative control.

4.2.7. Fluorescence microscopy

For microscopy, cells were stained with CYTO-ID® Red long-term cell tracer kit following manufacturer's instructions. Stained cells were included within Carbopol formulations and fluorescence (Exc = 561 nm, Emm = 575/25 nm) was analyzed after 24 h by using a microscope (Eclipse Ti2 Nikon, Tokyo, Japan) equipped with a Crest X-Light spinning disk confocal microscope and a Lumencor SPECTRA X light engine.

4.3. Results and discussion

4.3.1. Rheological characterization

The most common and affordable approach for 3D bioprinting was the extrusion-based technique². The capability of ink to be extruded was influenced by several factors including printing parameters, ink viscosity and shear thinning abilities. For instance, pressure and nozzle diameter could be modulated to facilitate printing and to enhance resolution. Then, the intrinsic mechanical properties of the formulations could be assessed by rheological analysis¹⁹. In this paragraph, the rheological properties of three different Carbopol formulations would be investigated. Specifically, the starting materials were supplied as a white and dry powder of primary particles averaging 0.2 μm in diameter which were sterilized by UV exposure for 30 minutes. The selected formulations were ETD 2020 NF and Ultrez 10 NF which were copolymers of acrylic acid modified with polyethylene glycol and a long-chain alkyl acid ester, and NF-980 which was a polyacrylic acid polymer without any chain modification (<https://www.lubrizol.com/Health/Pharmaceuticals/Excipients/Carbopol-Polymer-Products>). All the solutions were prepared at room temperature. Specifically, the powder was dissolved under continuous stirring in cell culture medium (i.e. RPMI 1640 or MEM) at specific concentrations: 1.2 w/v% for ETD 2020 NF and NF-980, 1.5 w/v% for Ultrez 10 NF. When complete dissolution was achieved, the solutions were neutralized by adding 5 M NaOH to reach maxim swelling.

First, the capability of the gel to accumulate (G' or storage modulus) or dissipate energy (G'' or loss modulus) had to be defined to understand if the elastic behavior overcame the viscous one ($G' > G''$) or viceversa. Importantly, for extrusion-based printing, bioinks had to show an elastic behavior²⁰.

Hence, the storage (G') and loss (G'') moduli of our formulations were obtained by oscillatory rheology as a function of shear stress. Interestingly, all of them exhibited elastic behavior (i.e. $G' > G''$), but, NF-980 showed a more viscous attitude than ETD 2020 NF and Ultrez 10 NF. Specifically, the approximate elastic moduli were $G' = 2,34 \times 10^2$, $3,57 \times 10^2$, $1,81 \times 10^2$, for ETD 2020 NF, Ultrez 10 NF and 980-NF, respectively (Fig. 4.1a).

Another important feature, which defined the printability of a gel was the shear thinning behavior. Precisely, the viscosity had to decrease upon stimuli application, allowing the flow of the ink through the nozzle when pressure was applied during the printing²¹. Importantly, all selected formulations showed a marked shear thinning behavior (Fig. 4.1b). Taken together, these results demonstrated that the ink could be printed. However, another important

parameter was the resolution post-printing. Indeed, the maintenance of the shape after printing was crucial for the establishment of useful scaffolds ²¹. To assess this property, a thixotropic curve was derived for each formulation, to mimic the application of the stress and all of them were able to return to rest condition after the stimuli disappearance (Fig. 4.1c).

To conclude, all Carbopol types could be hypothetically used as bioink. Importantly, mechanical parameters could not really predict cell viability after printing. Indeed, the residence time of the cells in the cartridge and the shear stress applied during the process could strongly influence cell survival. Importantly, shear stress could be modulated by changing nozzle size and shape, printing pressure and ink's viscosity.

Interestingly, some studies showed that below a critical shear stress level (about 10 kPa) cells can be dispensed without any damage ²², and, that moderate levels of shear stress might even stimulate cell proliferation, whereas this effect is reversed when this threshold is exceeded ²³. The performed rheological analysis suggested that the selected Carbopol formulations owned typical properties of a bioink but printing resolution and cell survival had to be assessed.

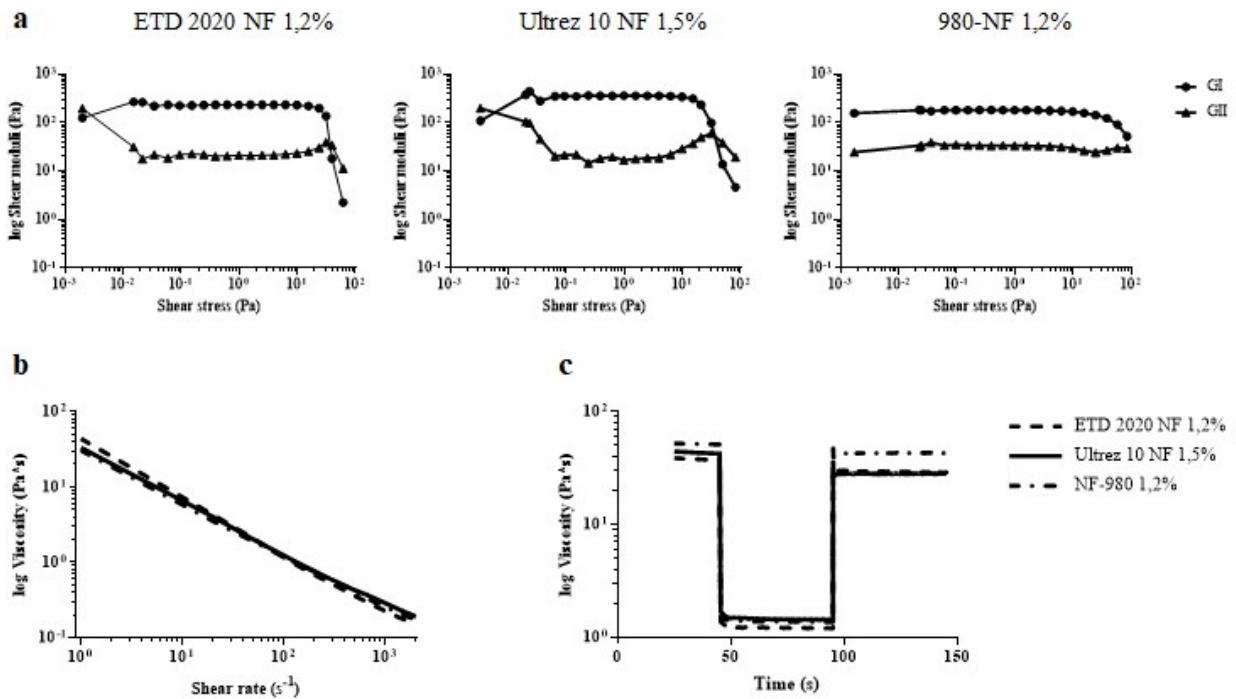


Fig. 4.1: Rheological analysis. a) Oscillatory tests in which shear stress ranged from 0,001Pa to 100 Pa while was kept constant at 1 Hz. The obtained curves represented the elastic (G') and the viscous (G'') moduli; b) Shear viscosity test to assess shear thinning behavior; c) Thixotropic curve to evaluate the recovery of properties at rest after the removal of the stimulus. Adapted from Baruffaldi et al., 2021 ¹.

4.3.2. Printing test

To evaluate Carbopol printability, the first step was the evaluation of the flow rate. Specifically, the amount of extruded inks was assessed at several pressures by using a conical 25G nozzle. As expected from rheological analysis, ETD 2020 NF and Ultrez 10 NF exhibited similar behavior, whereas NF-980 required higher pressures (i.e., from 20 kPa to 30kPa) to be extruded and the amount release was more difficult to be finely controlled (Tab. 4.1). These observations confirmed the previous analysis, specifically, the more viscous behavior of NF-980 formulation.

To evaluate the printing resolution of the three Carbopol formulations, a standard grid was printed with the BIOX bioprinter at the previously chosen pressures (Tab. 4.1). The grid measured 12 mm x 12 mm and the lines were set 3 mm apart. ETD 2020 NF and Ultrez 10 NF showed better results at lower pressure (13 kPa for ETD 2020 NF and 10–13 kPa for Ultrez 10 NF), while the grid printed with NF- 980 did not reach enough printing accuracy (Fig. 4.2). This analysis suggested that ETD 2020 NF and Ultrez 10 NF were the best candidate for the printing of cell-loaded bioink because of the lower printing pressure required which could be correlated to lower cell damage and death.

Bioink	Pressure (kPa)	Flow rate (mg/s)
ETD 2020 NF 1,2%	10	4,0
	13	9,9
	15	15,9
	20	31,7
Ultrez 10 NF 1,5%	10	5,6
	13	9,5
	15	12,6
	20	27,6
NF-980 1,2%	20	9,5
	23	22,6
	25	24,2
	30	37,8

Tab. 4.1: Flow rate of ETD 2020 NF, Ultrez 10 NF and NF-980 at different printing pressures using 3 mL cartridge with 25G conical needle.

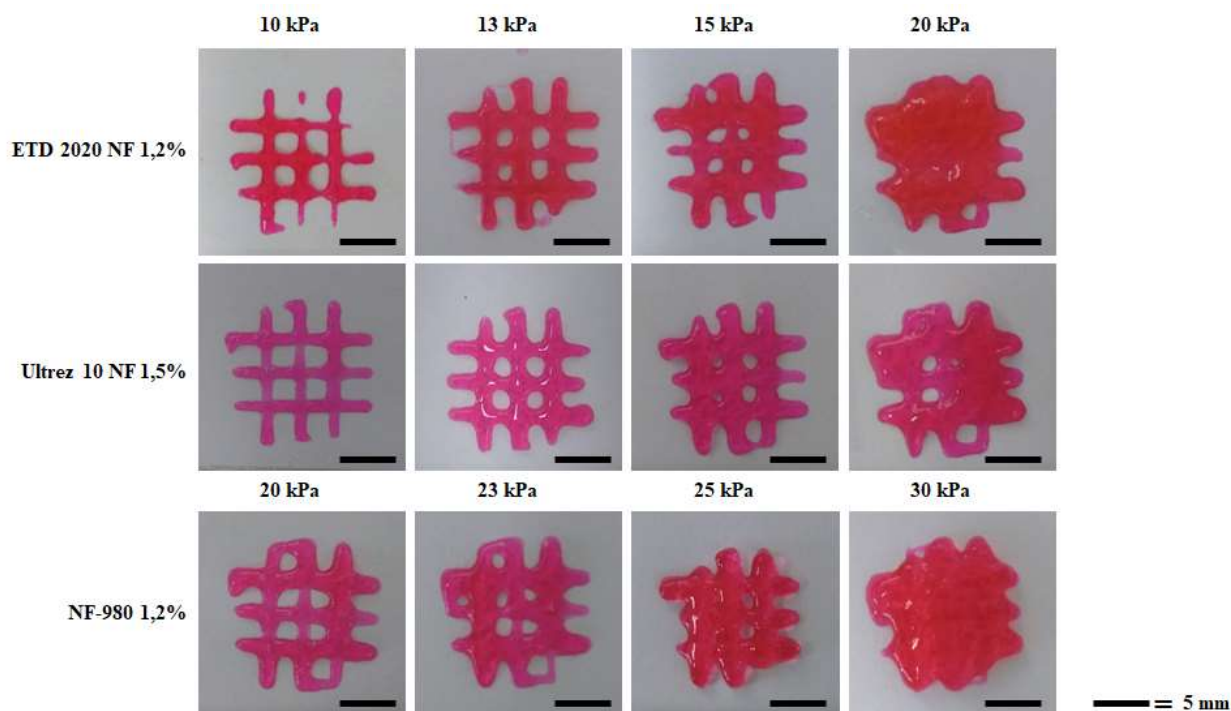


Fig. 4.2: Printing tests with BIOX bioprinter (CELLINK) at different pressures: 10-13-15-20 kPa for ETD 2020 NF and 20-23-25-30 kPa for NF-980. 3 mL cartridges equipped with 25G conical needles were used for all the tests. Adapted from Baruffaldi et al., 2021 ¹.

4.3.3. Cell viability analysis

Before the analysis of cell survival after printing, the cell toxicity related to Carbopol formulations had to be investigated. Although Carbopol did not present any cell-adhesive site, the capability of two different cell lines (A549, lung cancer epithelial cells, or MRC-5, normal lung fibroblasts) to grow on the material was evaluated. Specifically, 96-well plates were coated with a thin layer of printed bioinks and cells were seeded onto them. MTT analysis was performed 6 h and 24 h after seeding, and cells grown on non-coated wells were used as a positive control. Interestingly, both cell types succeeded in adhering to the Carbopol, but, after 24 h the growth was lower than the control. Moreover, A549 tolerated better than MRC-5 the presence of the material, apart from NF-980 which seemed to cause cell death after 24 hours (Fig. 4.3). To further confirm the adhesion, stained cells were visualized under a fluorescence microscope, confirming the results previously obtained (Fig 4.4).

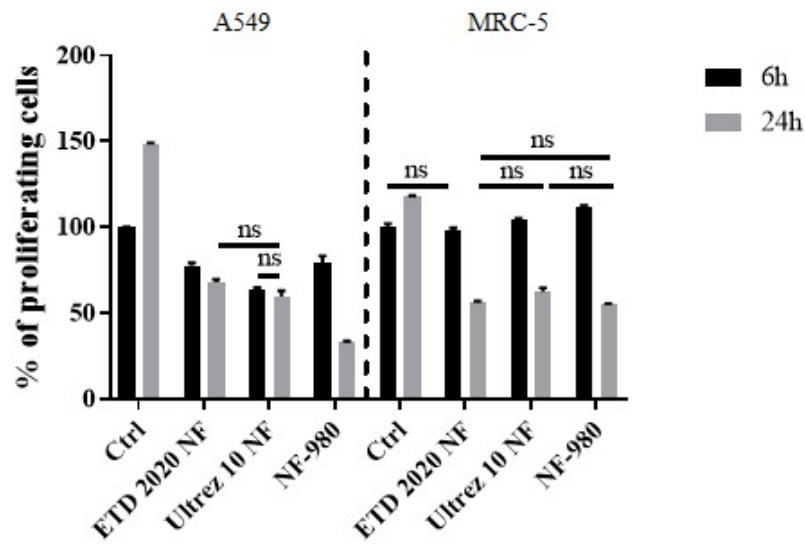


Fig. 4.3: MTT assay. A549 and MRC-5 were seeded on a thin layer of printed bioinks ETD 2020 NF 1,2%, Ultrez 10 NF 1,5% and NF-980 1,2% prepared in cell culture medium, RPMI 1640 and EMEM, respectively. The signal was analyzed 6 hours or 24 hours after seeding. Tests were performed at least three times and results are presented as the means $\pm$ SD. Only not significant (ns) differences were indicated.

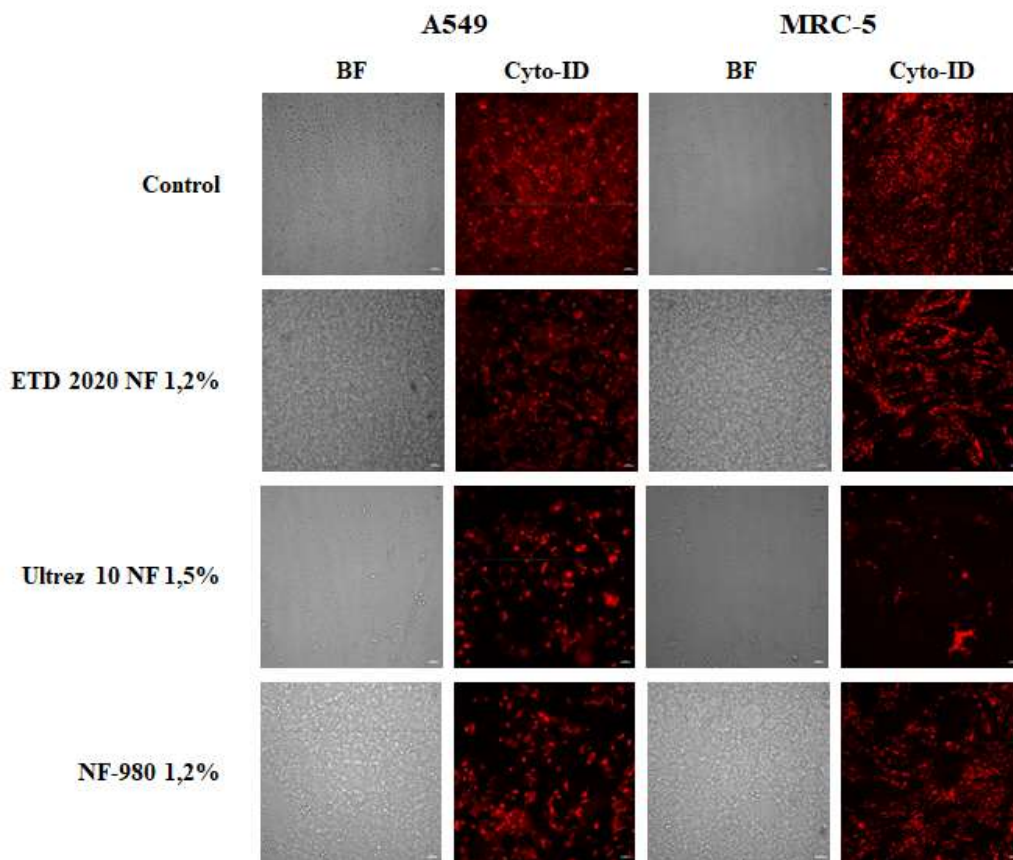


Fig. 4.4: Fluorescence images of stained A549 and MRC-5 growth on a thin layer of printed bioinks ETD 2020 NF, Ultrez 10 NF and NF-980, 24 hours after seeding.

Since the goal of the work was the development of a bioink, the capability of the loaded material to preserve cell survival played an important role. Hence, the optimal cell density had to be set. For this preliminary analysis, the experiments were performed by using the ETD 2020 NF which showed better results in the analysis described above (Fig. 4.3; 4.4). Importantly, since printing-related cell death could be relevant²⁴, two high cell densities were selected: 1×10^6 cells/mL and 3×10^6 cells/mL. As showed in Fig. 4.5, the higher amount of cells was linked to better performances in terms of cell survival, thus, the next experiments would be performed by using this density.

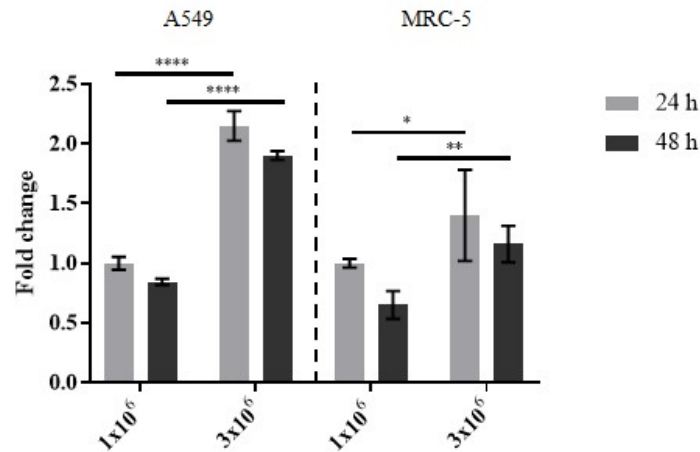


Fig. 4.5: MTT assay. A549 or MRC-5 were dispersed into ETD 2020 NF at two different densities: 1x10⁶ and 3x10⁶ cells/ml. The signal was analyzed after 24 h or 48 h of culture. Results are presented as mean±SD. *P<0,05, **P<0,01, ***P<0,001, ****P<0,0001

At this point, the next step was the evaluation of the effect of printing on cell proliferation. To do this, Carbopol formulations were filled with A549 or MRC-5 cells, and after that, the obtained bioinks were loaded into 3 mL cartridges equipped with 25G conical needle. The printing pressure was set at 13 kPa for ETD 2020 NF and Ultrez 10 NF and at 20 kPa for NF-980. The printing speed was kept constant at 16 mm/s. Then, viability was evaluated by using MTT assay immediately after printing (0 h) to assess printing-related death and 24 h later. Importantly, not-printed samples were used as a positive control. As expected, printing is associated with viability decrease as can be seen in Fig. 4.6. Interestingly, ETD 2020 did not show a significant difference at 24h both for A549 and MRC-5, but the latter seems to suffer less than the embedding into the material (Fig. 4.6a). On the other hand, Ultrez 10 strongly decreased A549 viability after 24 h also in the control suggesting some toxic effects of the material towards this cell line. Once more, MRC-5 tolerated better the material, and a slight viability decrease was observed only at 24h in printed samples (Fig. 4.6b). On the contrary, NF-980 strongly affected proliferation promoting cell death after 24h of culture also in the control samples (Fig. 4.6c). Taken together, these results suggested that ETD 2020 NF was the only matrix, which could be used for 3D printing without strongly affecting cell viability. To further confirm the presence of the cells inside ETD 2020 NF, the samples were analyzed under a fluorescence microscope (Fig. 4.7).

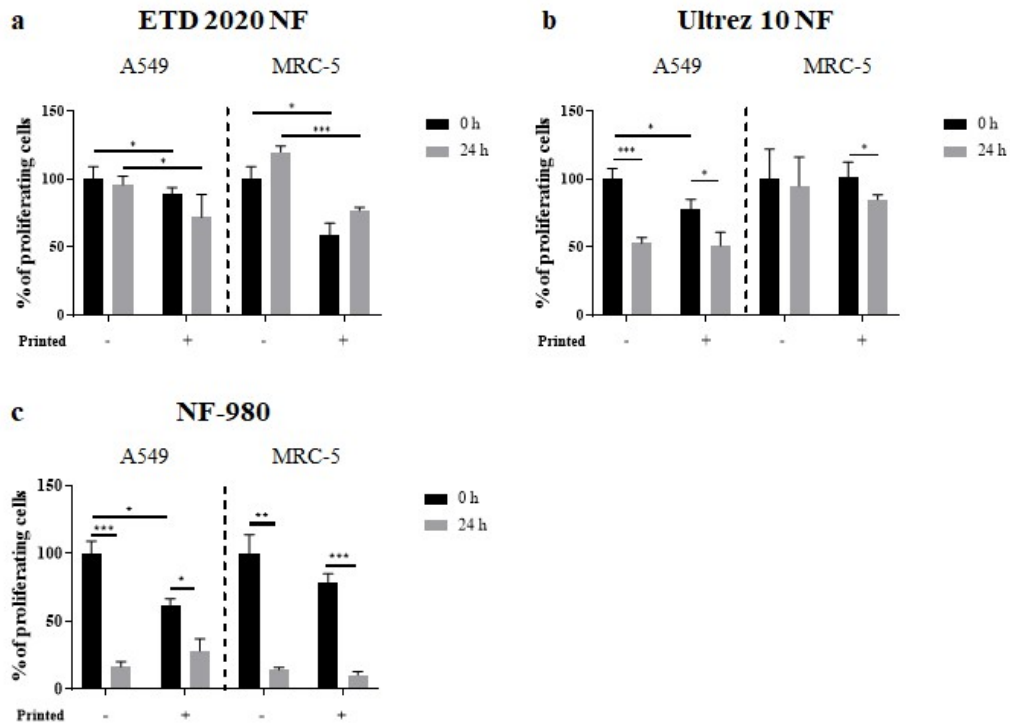


Fig. 4.6: MTT assay. A549 or MRC-5 were dispersed into ETD 2020 NF (a), Ultrez 10 NF (b) and NF-980 (c) at the density of 3×10^6 cells/ml. Printed (+) or not-printed (-) samples were tested. The signal was analyzed immediately after printing (0 h) and after 24 h of culture. Results are presented as mean $\pm$ SD. * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$, **** $P < 0,0001$. Adapted from Baruffaldi et al., 2020 ¹.

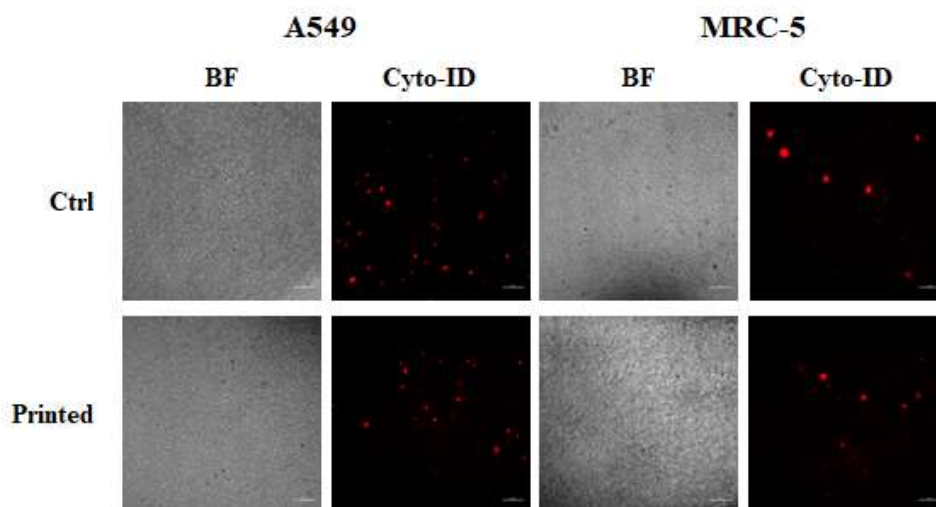


Fig. 4.7: Fluorescence analysis. Stained A549 or MRC-5 were included inside ETD 2020 NF at the density of 3×10^6 cells/mL. The samples were analyzed after 24 h printing. Adapted from Baruffaldi et al., 2020 ¹.

4.4. Conclusions

In this chapter, the successful preparation of three different formulations derived from three types of Carbopol was described. After the optimization of the preparation protocol, the rheological analysis demonstrated the feasibility of using them as inks for extrusion-based bioprinting. All of them were characterized by an elastic and shear-thinning behavior and after the application of a stimulus, they were able to recover the initial viscoelastic properties, suggesting the possibility of maintaining the shape after printing. Unfortunately, printing tests showed that NF-980 formulation required higher pressure to be printed and the resolution was worse than the one obtained with the other two preparations, ETD 2020 NF and Ultrez 10 NF. However, the analysis of cell adhesion was performed in all the formulations, specifically 96-well plate was coated with a thin layer of each preparation and, later, the cells were grown in 2D. Although, viability level was lower than in the control seeded on plastic, these results were quite expected due to the lack of cell adhesive moieties. However, promising levels of adhesion were reached, hence, the viability of cell included into the inks were evaluated and the effect of printing was also assessed. Interestingly, ETD 2020 NF and Ultrez 10 NF successfully supported cell survival even if, as expected, the printing process slightly decreased the cell viability. At the end of the study, NF-980 was excluded from further evaluation, because of the lower resolution and the higher printing pressure needed, that in turn decreased the cell survival rate after printing. Moreover, NF-980 presented a higher toxic effect on cells, preventing their growth. ETD 2020 NF showed better results in terms of printing and cell viability; thus, it was selected for further analysis. Although Carbopol lacked long-term stability and cell-adhesive motifs, it can be successfully employed as sacrificial ink during the printing of complex scaffolds that required internal empty channels. Indeed, the final goal of the project is the characterization of reliable NSCLC *in vitro* platform which present all the player involved in

tumor progression and metastasis formation. The use of a soft and easily removable ink during printing could be an effective strategy to obtain empty channels which will be colonized by endothelial cells to mimic vasculature contribution in the process.

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Chapter 5

Design of a 3D printed microfluidic platform for cell culture

Abstract

In this chapter, the design of a microfluidic platform will be described. In the first part, the use of a light-based 3D printing technique, called digital light processing (DLP), will be used to obtain 3D printed constructs from several acrylate based photocurable resins (i.e., polyethylene glycol diacrylate or PEGDA, 1,6-hexanediol diacrylate or HDDA, bisphenol A ethoxylate diacrylate or BEDA). The printing procedure, the post-curing and the washing steps have been deeply investigated to demonstrate the biocompatibility of the final constructs¹. Then, the analysis of the printability, biocompatibility, and molecule retention of an acrylate polydimethylsiloxane copolymer (TEGORad) was assessed, with the aim of finding an appropriate material for microfluidic device². At last, preliminary results about a multi material 3D printed 9-well plate will be discussed.

5.1. Introduction

Drug development is a long-term process, which requires several years to be completed. Indeed, after the identification of the target and the optimization of the active molecules, which involves a plethora *in silico* and *in vitro* analyses, a multitude of pre-clinical (i.e. *in vivo* models) and clinical tests have to be performed³. It is clear that a high-throughput screening process optimizes the selection of promising molecules. However, cell-based analyses have to take into account the response of complex systems, like organs, to fully characterize the selected molecules⁴. For instance, in the development of anti-tumoral drugs, the role of cancer cells and the microenvironment have to be analyzed⁵. Hence, the development of microfluidic devices, which reproduce fluid flow, pressure and chemical gradients could speed up the process. Indeed, the possibility to mimic the interaction and the paracrine signaling that occurs *in vivo* improves the prediction of drug effects in this complex environment during cancer progression and drug treatment⁶. Microfluidic technologies have implemented several processes thanks to the decrease of reagent and time required for the analysis, the reduction of waste and the successful modeling of physiological conditions in 3D cell culture⁷.

Importantly, microfluidic device could be produced through several processes: among those, 3D printing based technology leads to improvement in terms of resolution, easy-handling, and versatility. Interestingly, the main used techniques rely on the use of photocurable resins (e.g., stereo lithography appearance, or SLA, and digital light processing, or DLP)⁸. The main limitation of this approach is the lack of photosensitive formulations, which usually includes acrylate, that satisfy the need of biodegradation and biocompatibility required for biological research⁹. Further, also the selection of the optimal photoinitiators required several analyses. Indeed, it must have

high quantum efficiency, thermal and temporal stability and the absorption wavelength has to be the one of the light sources. It must have good solubility in the final formulations, and it has not to cause yellow coloration in the cured products, finally, harmful effects on cells must be avoided ¹⁰.

For this reason, mechanical properties, printability, and biocompatibility of different acrylate formulations were deeply investigated. PEGDA, HDDA, and BEDA monomers have been widely used for DLP-based printing ^{11,12}. Further, the amount of photoinitiator and dye were modulated to find the better condition for the establishment of cell culture. The aim of the project was the development of a microfluidic platform for the culture of NSCLC models (i.e. co-culture and spheroids previously described) to automatize the process of drug testing and the analysis of molecular features of patient-derived specimen.

5.2. Materials and methods

5.2.1. Materials

Polyethylene glycol diacrylate (PEGDA, Mn = 250 g/mol), 1,6-hexanediol diacrylate (HDDA, Mw = 226.3 g/mol) and bisphenol A ethoxylate diacrylate (BEDA, Mn ~ 512 g/mol, EO/phenol 2) were selected as diacrylate monomers and they were all purchased from Sigma Aldrich. Instead, TEGO®Rad 2800 (TEGORad) was kindly supplied by Evonik Industries AG (Essen, Germany). Phenyl bis(2,4,6-trimethylbenzoyl) phosphine oxide (BAPO), 2-Hydroxy-2 methylpropiophenone (HMP), Dansyl Chloride, Methyl Methacrylate (MMA) were also purchased from Sigma Aldrich. As described in previous chapters, tumor cell lines A549 were kindly provided by Valentina Monica from University of Turin, whereas human keratinocytes (HaCaT) were obtained from Antibody Research Corporation. For the analysis of PEGDA, HDDA and BEDA biocompatibility, RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin (all from Sigma Aldrich) and glutamine (from Biowest) were used. For TEGORad analysis, BenchStable™ RPMI 1640 and BenchStable™ DMEM were purchased from Thermo Fisher Scientific. Moreover, as mentioned in the previous chapters, MTT assay (M2003) and Live/Dead staining kit (04511-1KT-F) were bought from Sigma-Aldrich as well as DAPI, FITC-conjugated Phalloidin and PrestoBlue™ Cell Viability Reagent.

5.2.2. Formulations preparation

PEGDA, HDDA and BEDA formulations were prepared by adding 0.2% or 1% of BAPO photoinitiator into each acrylate monomer. To obtain complete dissolution, the mixtures were sonicated at least for 30 minutes at room temperature (RT).

For TEGORad formulation, BAPO was solubilized in HMP at the ratio BAPO:HMP of 1:4. Then, the dye Dansyl Chloride was dispersed into MMA at the weight ratio of 1:50, and then, added to TEGORad and BAPO mixture

at different concentrations: 0.075 w/v%, 0.05 w/v %, 0.01 w/v %. To reach homogeneity, the formulations were magnetically stirred and sonicated for 5 minutes at RT.

5.2.3. Digital Light Processing (DLP) 3D printing

A PICO 2 DLP-3D printer (Asiga, Australia) was used for processing of the acrylate-based formulations (i.e., PEGDA, BEDA and HDDA). This 3D printer has a LED light source (405 nm) with a nominal XY pixel resolution of 50 μm , whereas the minimum Z-axis control is 1 μm . Instead, MAX X27 UV DLP-3D printer (Asiga, Australia) was used for processing TEGORad formulations and the multi-materials plate. The light source emits at 385 nm and it has a nominal XY pixel resolution of 27 μm and Z-axis control of 1 μm .

5.2.4. Washing steps, post-curing and sterilization

To optimize the cytocompatibility, the 3D printed samples obtained from each formulation, were deeply washed. Specifically, PEGDA, BEDA and HDDA samples were washed with ethanol or acetone for the complete removal of unreacted products. Four different washing protocols, identifiable into two main groups, were tested (Tab. 5.1). Particularly, the protocols were grouped as follows:

- 1) the samples were sonicated for 5 minutes in a solvent (ethanol or acetone), followed by 5 minutes of UV post-curing for the inside and 5 minutes for the outside of the wells. After the post-curing, the samples were kept in the indicated solvent overnight.
- 2) the samples were incubated for 2 hours in the indicated solvent (ethanol or acetone), then UV post-cured for 5 minutes for the inside and 5 minutes for the outside of the wells.

Washing protocol		5 minutes sonication	2 hours incubation	UV post-curing	Overnight incubation	H ₂ O	Sterilization
1	SON Acetone	✓ (Acetone)		✓		✓	✓
2	SON EtOH	✓ (Ethanol)		✓		✓	✓
3	INC Acetone		✓ (Acetone)	✓	✓ (Acetone)	✓	✓
4	INC EtOH		✓ (Ethanol)	✓	✓ (Ethanol)	✓	✓

Tab. 5.1: Schematic representation of washing protocols.

Then, three different sterilization methods were performed: UV light for 30 minutes inside a biosafety cabinet, immersion in 70% ethanol for 10 minutes then air-dried at RT or autoclaved for 20 minutes at 121°C.

Instead, TEGORad printed devices are placed in acetone for 20 minutes. Then, the solvent is refreshed, and the samples are left overnight in the same solvent. The next day, a further change is carried out and kept for another 20 minutes in immersion, then raised again and dried. All these steps were done in the dark. At this point, the 3D

printed objects underwent to a post-curing treatment in a UV oven to complete the crosslinking (5 minutes each side). Then, they are immersed in ultra-pure water and, sterilized by autoclave process. Finally, they are left to dry at RT for 24 h.

The washing protocol of multi-material samples consisted in 30 minutes of incubation in acetone, solvent refreshing, then other 30 minutes of immersion. Then, samples were incubated overnight in ethanol. To complete the crosslinking, ethanol was diluted with deionized water and then the samples were rinsed and post-cured in a UV oven for 5 minutes each side.

5.2.5. Rheological analysis

Viscosity of the formulations were measured using an Anton Paar rheometer (Physica MCR 302) at a constant temperature of 25 °C and in parallel-plate mode with a diameter of 20 mm and a gap of 50 µm. The shear rate range was set up from 1 to 100 s⁻¹. Real-time photo-rheological tests were carried out using a 20 mm quartz lower parallel plate and a 20 mm aluminum upper parallel plate; a Hamamatsu LC8 lamp equipped with a visible bulb and a cutoff filter below 400 nm and an 8 mm light guide was using to study the photopolymerization process. During the tests, the light irradiation with an intensity set at 20 mW/cm² initiated after 60 seconds. The measurements were performed at a constant temperature of 25 °C, at constant strain amplitude of 1%, and a constant shear frequency of 1 rad/s.

5.2.6. FTIR analysis

To assess the acrylate conversion on liquid resins and 3D printed construct, a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, Milano, IT) was used. The spectra were recorded using an attenuated total reflectance (ATR) accessory (Smart iTX). Specifically, the spectra were collected with a resolution of 4 cm⁻¹, averaging 32 scans for each spectrum, wavenumbers range 650 – 4000 cm⁻¹. The conversion of the acrylate double bond (C=C) was monitored by the scissoring acrylate vibration at 1409 cm⁻¹. The area of the acrylate peaks was normalized by the carbonyl (C=O) constant signal centered at 1725 cm⁻¹.

5.2.7. Transmittance

TEGORad samples were put in a 12 well plate and the absorbance of the material was analysed by using Synergy™ HTX Fluorescence Multi-Mode Microplate Reader. The transmittance was calculated as follows:

$$T = e^{-A} * 100$$

Where $A = \frac{(A_{12\text{ well}} - A_{\text{pol}})}{1\text{ mm}}$ * 0.250 mm since the TEGORad wells ($A_{12\text{ well}}$ = TEGORad absorbance) were put in a polystyrene well plate (A_{pol} = Polystyrene absorbance) and the thickness of the bottom was 1 mm for the TEGORad wells and 250 µm for the polystyrene well plate.

5.2.8. *In vitro* cell culture

For cell culture analysis, lung cancer epithelial cells A549 were maintained in RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM glutamine or BenchStable™ RPMI 1640 that does not required glutamine adding. Normal epithelial cells, named HaCaT, were cultured in BenchStable™ DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate and 2% L- glutamine.

5.2.9. Proliferation and viability analysis

To test the biocompatibility of different formulations of PEGDA, HDDA and BEDA, 96-plate like wells were used for cell culture (inner diameter= 5,6 mm). For MTT assay, 1.5×10^4 A549 were seeded on each sample and incubated in complete medium at 37°C. MTT analysis was performed after 48 h and 96 h of culture. It is used at the concentration of 0,5 mg/mL MTT. After 2 h of incubation at 37 °C, MTT solution was removed and 250 ul of MTT solvent (10% SDS, 0,01 M HCl in H₂O) was added to solubilize the formazan crystals and the plate was incubated for 2 h at 37°C. Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winoosky, Vermont, USA) was used to read the optical density (OD) at wavelength of 570 and 650 nm.

3D-printed PEGDA samples were also analyzed by live and dead assay (Sigma-Aldrich). Specifically, cells were washed 3 times with PBS, then they were stained with 1,5µM Propidium Iodide and 1µM Calcein-AM for 15 minutes at RT. Fluorescence was detected with a spinning disk confocal microscopy system. Cells grown on conventional polystyrene (PS) wells were used as positive control.

5.2.10. Conditioned medium experiment

Cell culture media (BenchStable™ RPMI 1640 or DMEM) were conditioned with sterilized TEGORad samples for 72h at 37 °C. Then, 1×10^4 A549 or HaCaT were seeded onto a 96 well plate and grown in conditioned medium (CM). After 24 h and 72 h PrestoBlue™ Cell Viability Reagent was added at a concentration of 10% v/v and was incubated for 1 hour at 37°C. Then the supernatant was transferred and the fluorescence was measured using Synergy™ HTX Fluorescence Multi-Mode Microplate Reader with the excitation/emission wavelengths set at 530/590 nm.

5.2.11. Cell staining

To visualize cells on TEGORad surface, 48 well-lile TEGORad wells with 0.01, 0.05 or 0.075 w/v% dye were printed. To promote cell adhesion, samples were placed on a low-pressure plasma polymerization system (IONVAC Process s.r.l.) and treated with O₂ plasma for 1 minutes, at a pressure of 30 mTorr, 50 W and a flow rate of 10 sccm. After sterilization, 3×10^4 A549 or HaCaT were seeded onto 3D printed TEGORad wells in 400 µL/well of complete medium. After 72 h cells were fixed with 4% Paraformaldehyde at RT for 15 min and washed twice with DPBS.

For the staining, cells were incubated with 0.4 μM DAPI and 0.25 μM FITC-conjugated phalloidin in PBS (200 μL) for 30 min at RT. The samples were imaged with the spinning disk confocal microscopy system (Nikon) in the DAPI and FITC channels.

5.2.12. Drug-like test in microfluidic devices

The absorption of molecules by 3D printed TEGORad microfluidics was investigated by using a drug-like substance (Rhodamine 6G) used to test material absorption. For this purpose, 3D printed microfluidics made of different formulations have been designed. Afterwards, the devices have been sealed by bonding to a glass microscope slide. Irreversible bonding was achieved by treating both surfaces with O₂ plasma treatment.

PDMS devices were used as a positive control. A solution of Rhodamine 6G (1 μM in PBS) was prepared through sonication and then it was pumped through the devices. After 1 h, the constructs were washed with PBS overnight. The next day, the microfluidics were imaged with a Nikon Spinning disk confocal microscope and a fluorescent signal was detected.

5.3. Results and discussion

5.3.1. Characterization of BEDA, PEGDA and HDDA formulations

To test the feasibility of using acrylate based formulations for DLP-based printing, preliminary analyses were performed, in particular information regarding resin behavior, like viscosity, reactivity, and degree of conversion, were fundamental in the establishment of printing parameters. Indeed, viscosity has been shown to strongly influence material photopolymerization, knowing that lower values were associated with higher printing resolution^{13,14}. For this reason, choosing the optimal printing parameters required knowledge about viscoelastic properties of the resins. The lower values were found in PEGDA and HDDA formulations, whereas the BEDA resulted to be the most viscous. Nevertheless, all the solutions resulted to be suitable for DLP-based printing.

Then, a commonly used photoinitiator type I, called phenylbis(2,4,6-trimethyl-benzoyl) phosphine oxide (or BAPO) was selected, because its absorption even in the visible light¹⁵. Since BAPO could show some toxic effects on cells¹⁶, different percentages of photoinitiator were tested, so to decrease as much as possible its amount in the final formulation, leaving its radical forming ability. (Tab. 5.2).

Composition	Nomenclature	Viscosity (Pa · s)	Exposure time (s · layer)	Initial exposure time (s · layer)	Acrylate conversion (%)	Insoluble fraction (%)
PEGDA + 0,2 w/v% BAPO	P-0.2	0,018	1,5	3	88	98
HDDA + 0,2 w/v% BAPO	H-0.2	0,019	1,5	3	85	99
BEDA + 0,2 w/v% BAPO	B-0.2	0,071	2	3	72	95
PEGDA + 1 w/v% BAPO	P-1	0,018	1	2	89	99
HDDA + 1 w/v% BAPO	H-1	0,020	1	3	89	99
BEDA + 1 w/v% BAPO	B-1	0,073	1,5	2	78	96

Tab. 5.2: Nomenclatures and properties of PGDA, HDDA and BEDA formulations. Modified from Gonzalez et al., 2020.

Then, photorheological analyses were used to assess the viscoelastic properties, useful to determine printing parameters, so that the time required to complete the polymerization was essential to decide the printing rate and the light exposure. Thus, the storage moduli (G') during visible light irradiation were analyzed. As shown in Fig. 5.1a-b, all formulations were characterized by fast polymerization, nearly 1 second. Further, as expected, samples with lower amounts of BAPO required, a longer exposure time to complete the reaction. These results showed that during the printing process, the latter solutions required longer time to cure a layer of equal height than those with a higher amount of photoinitiator.

After these preliminary characterizations, printing tests with the three selected acrylate based formulations have been conducted. Since our goal was the production of a device for cell culture a CAD that resembled the structure of 24-plate wells was designed. In this way, the printed platform could perfectly fit in traditional instruments employed for biological research (e.g., plate reader, confocal microscope). As shown in Fig 5.2a, wells easily to be handle and transparent were obtained. Samples with a higher amount of BAPO showed a yellowish appearance (Fig. 5.2b), related to the unreacted BAPO in the material, that has absorption peak in the range 350 -420 nm (Fig. 5.1c), Importantly, all the samples presented high bottom transparency (Fig. 5.2b), which was essential for checking cell vitality and proliferation, through optical microscopy or the classic spectroscopic based biological assay. Further, the 3D printed devices were also analyzed by ATR-FTIR technique, to assess the decrease of the acrylate double bonds at 1409 cm^{-1} . As shown in Tab. 5.2, samples with higher amount of BAPO reached the higher degree of conversion, due to the greater generation of free radicals.

The fraction of unreacted monomers and photoinitiator was also evaluated through chloroform extraction of the insoluble components. The quantities were quite high for all the samples (Tab. 5.2) and BAPO presence, especially in B-1, was confirmed by absorbance analyses (Fig. 5.1d).

These results demonstrated the importance of developing post-processing steps for the 3D printed device to remove all unreacted species which could be toxic towards cells.

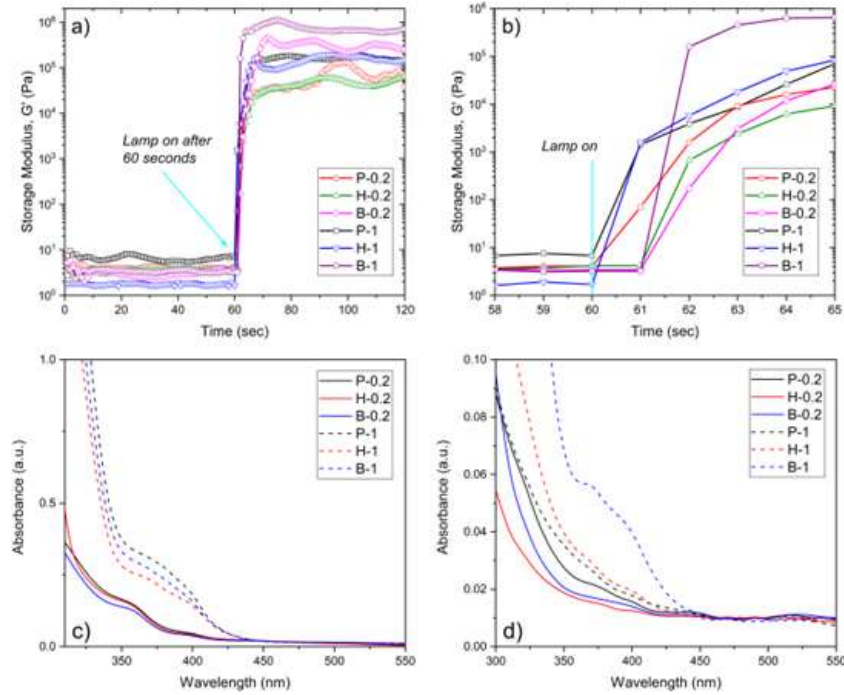


Fig. 5.1: a) Photo-rheological analysis of different PEGDA, BEDA and HDDA formulations containing 0.2 and 1 wt.% of BAPO. b) Zoom of the G' in the interval when the lamp was turned on. c) UV-vis spectra of 100 μm thick 3D printed discs d) UV-Vis spectra of the extracting solvent used for the calculation of the insoluble gel fraction of the 3D printed parts. Adapted from Gonzalez et al., 2020.

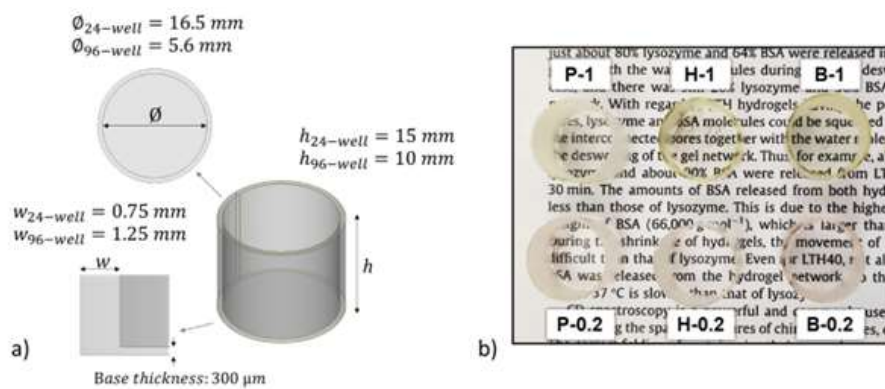


Fig. 5.2: a) View of the CAD designs showing the printed 24- and 96-like-well b) Picture of 3D printed 24-well-like samples. Adapted from Gonzalez et al., 2020.

5.3.2. Optimization of washing steps and sterilization methods

As described, the unreacted species could have an impact on cell adhesion and viability. For this reason, we assessed several washing protocols to remove all toxic species present in the printed constructs. First, we analyzed the effect of washing steps on the physical appearance of the different samples, remarking that integrity and transparency were fundamental properties of cell attachment and analysis. As presented in Fig. 5.3, sonication or incubation in acetone strongly impacts the structure of the printed disks, especially of samples made of BEDA and HDDA. Instead, ethanol-based washing seemed gentler and preserved more the integrity of the structure.

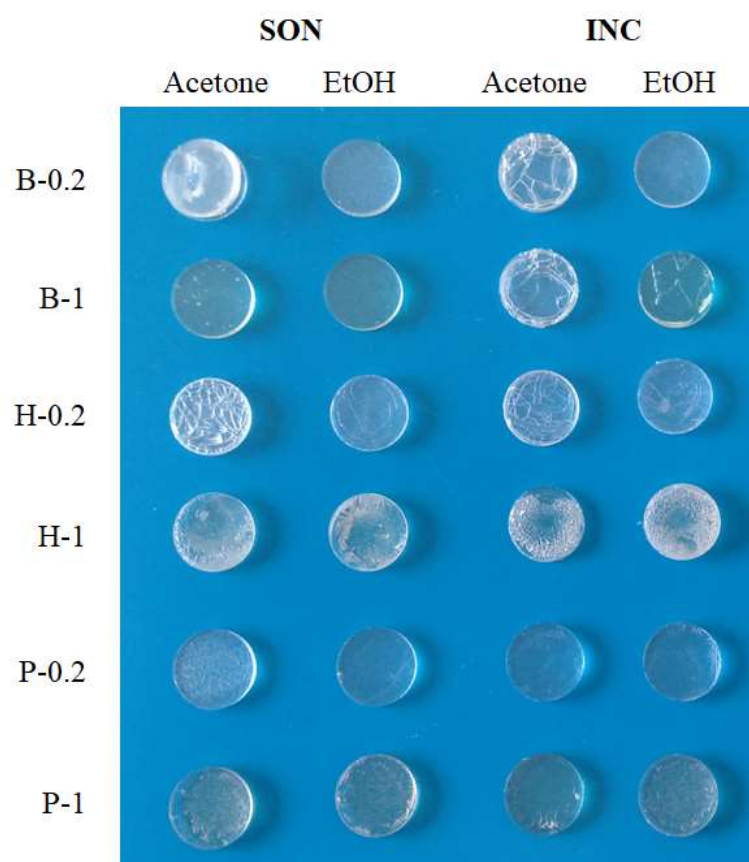


Fig. 5.3: Picture showing the optical and physical properties of 3D printed disks of all the formulations treated with different washing protocols. Adapted from Gonzalez et al., 2020.

To better evaluate cytocompatibility of the materials, a proliferation rate assay of cells grown on 96-well like construct treated with different washing protocols was assessed. For this analysis, all the samples were sterilized with UV irradiation for at least 20 minutes and cells grown on traditional plate were used as a positive control. As shown in Fig. 5.4, cell proliferation rate was strongly influenced both by materials and washing protocol. Unfortunately, BEDA samples failed in sustaining cell viability and proliferation (Fig. 5.4a), whereas HDDA (Fig.

5.4b) and PEGDA (Fig. 5.4c) showed better results. Although, HDDA supports a good level of cell proliferation in samples washed through the sonication-based protocol, the lack of transparency and the wide disruption of the disks after the treatments prevent its use in assays that include microscope analysis. Instead, all PEGDA samples showed no big differences in A549 proliferation after 48 h, independently of BAPO concentrations. Those results demonstrated the efficiency of our washing protocols in removing all unreacted BAPO from the printed constructs. However, we selected P-0.2 as formulation of election to minimize the risk of possible BAPO toxicity. Further, sonication-based washing in ethanol resulted in the better in terms of transparency, integrity, and cell toxicity (Fig.

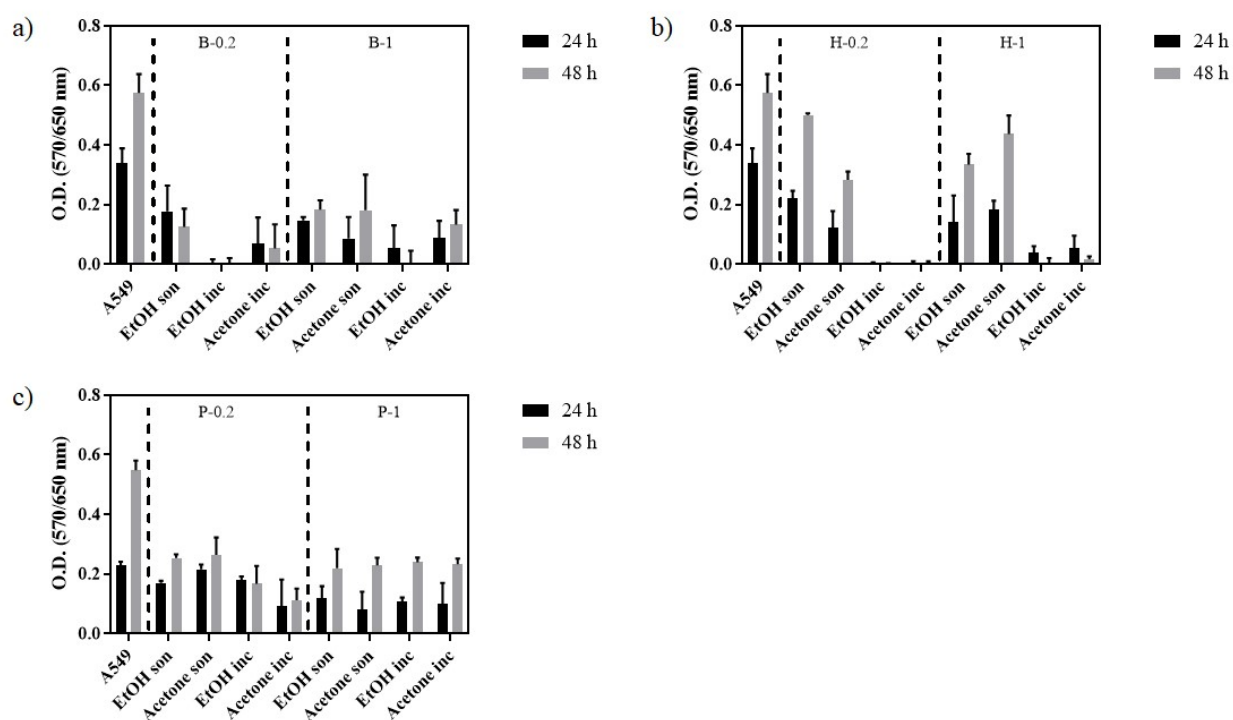


Fig. 5.4: MTT analysis on A549 cells seeded on different formulations made of BEDA (a), HDDA (b) or PEGDA (c). The viability was measured after 24 h or 48 h from seeding. Results were shown as mean $\pm$ SD. Adapted from Gonzalez et al., 2020.

5.3; 5.4c).

Next, cell viability on P-0.2 samples could be improved by optimizing the sterilization protocol. First, as shown in Fig. 5.5, wells structure maintenance was assessed after UV-exposure, autoclave, and ethanol immersion. As expected, UV-based sterilization succeeded in preserving the integrity. Then, cell viability in samples incubated or not in deionized water before the seeding was analyzed. Interestingly, viability was strongly improved by this step suggesting the removal of ethanol residues after the washing steps that could decrease cell attachment and proliferation (Fig.5.5). At the end, P-0.2 samples, washed with sonication-based method in ethanol, sterilized by

UV irradiation and incubated in deionized water before seeding was selected as the condition of election for further analysis.

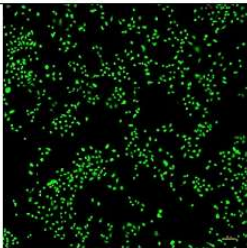
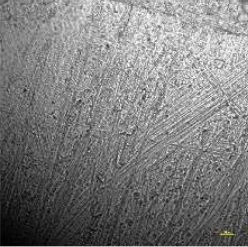
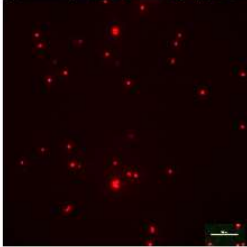
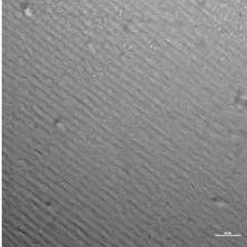
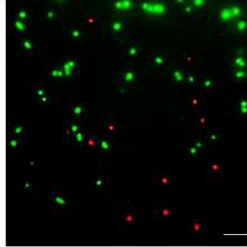
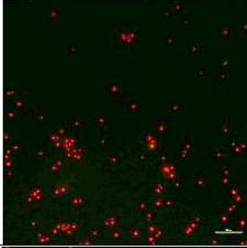
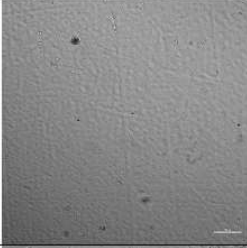
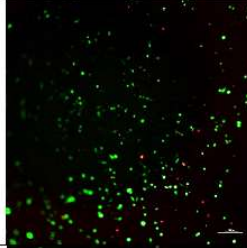
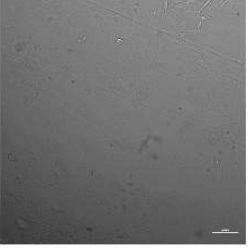
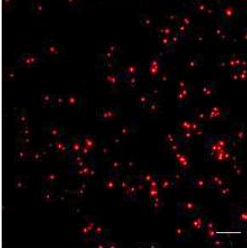
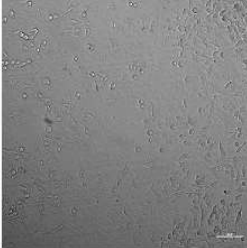
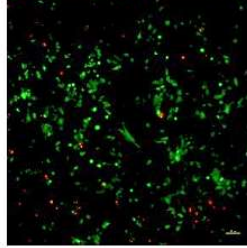
	Brightfield	Calcein AM/PI	Brightfield	Calcein AM/PI
	No H ₂ O treatment		+ o/n H ₂ O treatment	
Control				
Autoclave 				
Ethanol 				
UV light 				

Fig. 5.5: Picture of the real 3D printed wells sterilized by autoclaving, ethanol immersion and UV light exposure. Live/dead analysis were performed after 24h from the seeding. The scale bars denoted 100µm. Adapted from Gonzalez et al., 2020.

5.3.3. Characterization of TEGORad cytocompatibility

At this point, P-0.2 formulation was selected to be the main component of the microfluidic platform for cell culture for its printability, integrity post-washing, transparency and cytocompatibility. Hence, a resin suitable for the addition of channels, which could connect different wells, was investigated, in particular the main requirements were high resolution, transparency, harmless for the cells and not absorption of protein and molecules. Previous studies focused on the characterization of a printable acrylate PDMS copolymer, named TEGORad¹⁷. Indeed, PDMS, a silicone elastomer, was already investigated as starting material for microfluidic devices thanks to its cheapness, cytocompatibility, optical transparency, flexibility, chemical stability, and gas permeability¹⁸. Although its many advantages, it showed several limitations: the reproducibility which is manufacturer dependent, the time required for the thermal-driven polymerization, material wasting and the expensiveness¹⁹. Hence, since TEGORad is the DLP-printable form of PDMS¹⁷, it could represent the right material for microfluidic part of our device.

Since transparency remained a necessary requirement to monitor the cells, it was analyzed in different printable formulations, which contained both the previously described photoinitiator and a dye, called Dansyl Chloride, at different concentrations. Indeed, dye adding improve the resolution of the printed objects by avoiding the photopolymerization of the region next to the irradiated layer²⁰. Importantly, TEGORad resulted in an optically transparent material, especially for wavelengths above 470 nm, even in presence of dye (i.e. transmittance % $\geq$ 95% for of all the dye concentrations tested) (Fig. 5.6). This suggests that the monitoring of the cell culture on TEGORad devices is possible, even for the formulation with the higher concentration of dye.

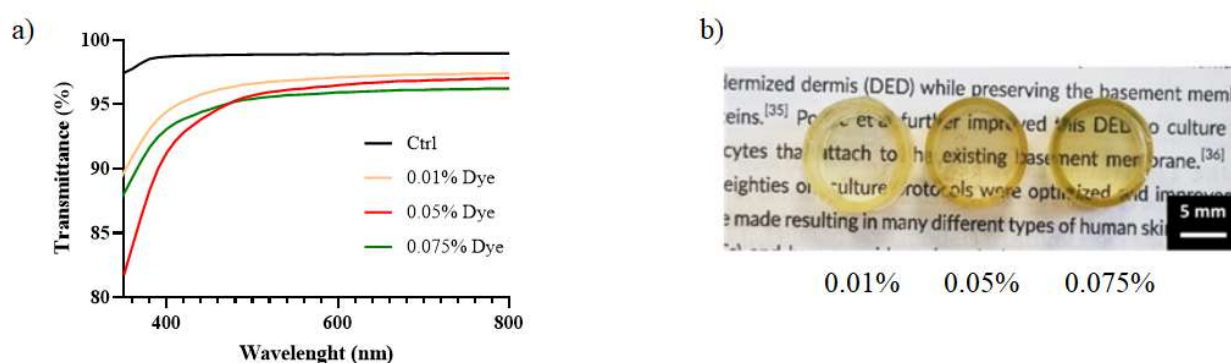


Fig. 5.6: a) Transmittance (%) in UV-visible spectra of TEGORad formulation with different dye percentage compared with the one of traditional polystyrene well (Ctrl). b) Picture of printed TEGORad wells.

The next step was the evaluation of different TEGORad formulations' cytotoxicity. Media conditioned by different printed samples were used to grow cells. As shown in Fig. 5.7, the proliferation rates of HaCaT (Fig. 5.7a) and A549 (Fig. 5.7b) was significantly decreased by the presence of a conditioned medium after 3 days of culture. The

noxious effect was more evident in normal epithelial cells (HaCaT) than in tumor cells (A549), suggesting a line-dependent response to the molecules released by the printed constructs. Although that, the slight viability decrease was compatible with the establishment of cell culture, hence, TEGORad formulations were kept into consideration for further analysis. Thus, to confirm its suitability for cellular analysis, adhesion tests were performed. As shown in Fig. 5.8, A549 and HaCaT successfully grew on TEGORad wells validating the previous described results. Taken together these outcomes proved the biocompatibility of the formulation, thus, it will be implied in the future development of the microfluidic platform for lung cancer studies.

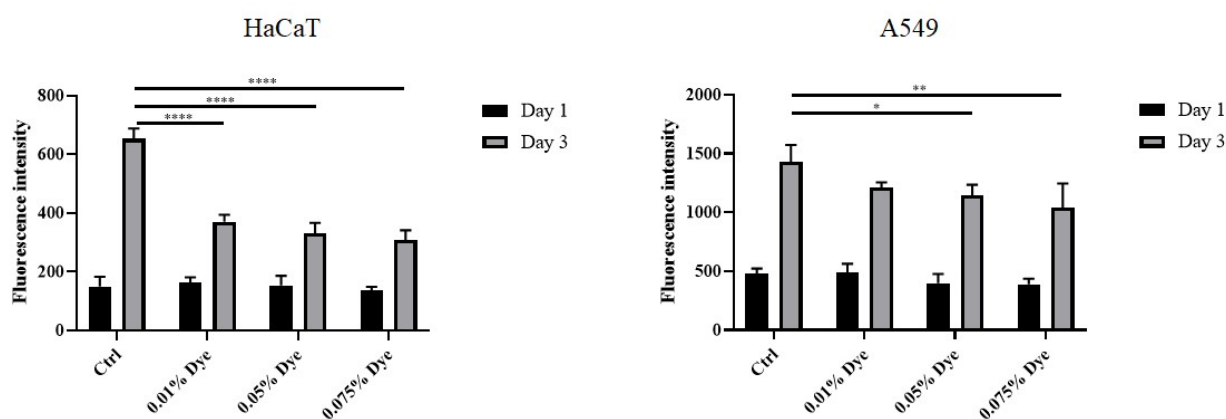


Fig. 5.7: MTT analysis on HaCaT grown with medium conditioned with different printed TEGORad samples. Cells grew with normal medium were used as control (Ctrl). Results were shown as mean $\pm$ SD. * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$, **** $P < 0,0001$

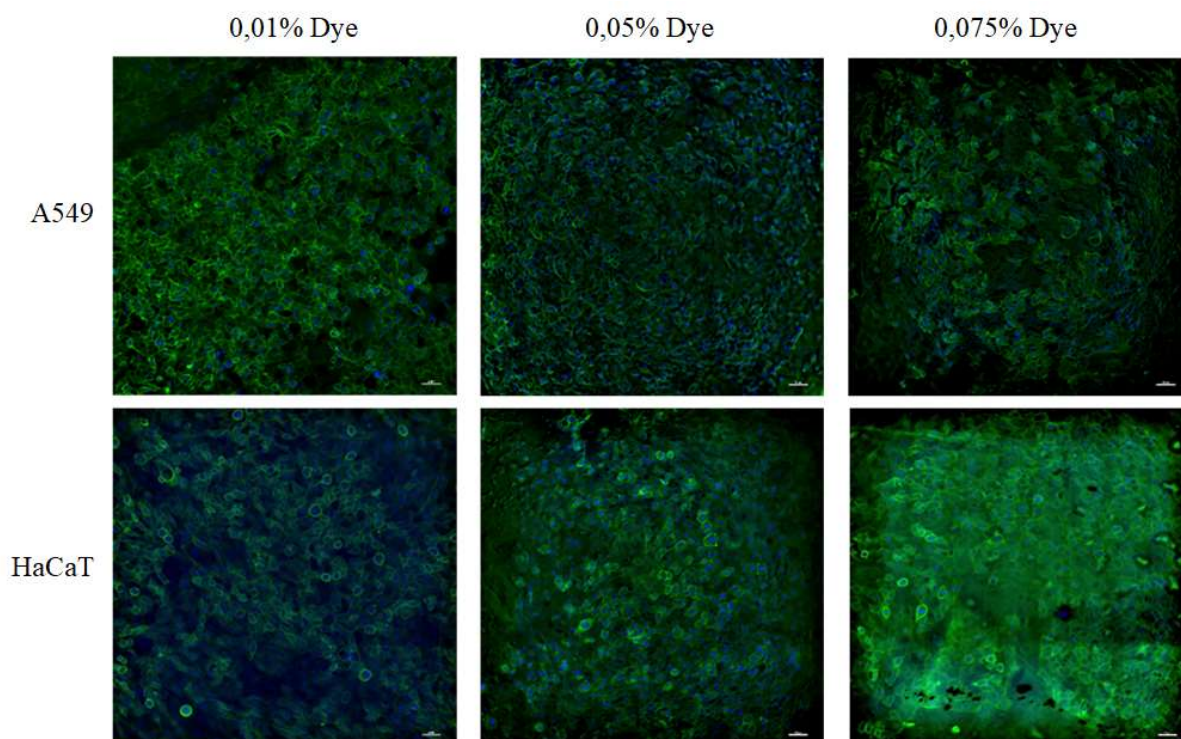


Fig. 5.8: Representative images of A549 and HaCaT grew on TEGORad wells. Cell's cytoskeleton was stained by Phalloidin (green signal), whereas nuclei by DAPI (Blue signal).

5.3.4. Assessment of molecules absorption by TEGORad constructs

The aim of this project was the printing of a microfluidic device for 3D cell culture as a platform for drug testing. Hence, the retention of molecules by the material and above all in microfluidic had to be avoided. It is known from the literature that PDMS presents high absorption of small hydrophobic molecules, including drugs ²¹, so this behavior was also evaluated in 3D printed TEGORad devices. To do this, TEGORad opened microfluidics were

printed and sealed with a glass slide thanks to the O₂ plasma treatment (Fig. 5.9a), while PDMS was used as a positive control. For the analyses, Rhodamine 6G was used as drug-like molecule and its retention was assessed by monitoring its fluorescence. Surprisingly, TEGORad absorption was significantly lower than the one found in PDMS (Fig. 5.9b). These results clearly demonstrated the feasibility to use TEGORad as main material in device for drug-testing, indeed the risk of hydrophobic molecules retention and changing in drug concentration over the experiments was decreased than in PDMS microfluidics.

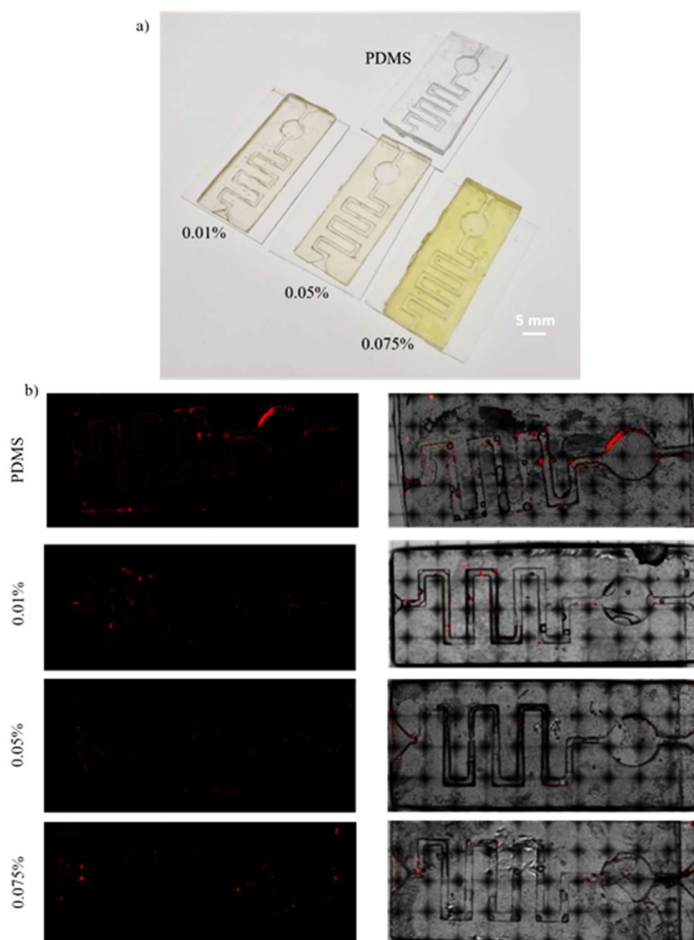


Fig. 5.9: a) Pictures of the microfluidics in PDMS Silgard 184 and three 3D printed TEGORad samples and b) fluorescence images of microfluidics after the flow of Rhodamine 6G and the washing protocol.

5.3.5. Printing and optimization of a multi-materials plate

At this point, preliminary tests were performed to print a plate in which the wells, which were designed to recapitulate the dimension of a 96-multi well plate, were connected by channels. Importantly, dimension and distance of the wells maintained the proportion of the traditional plate to avoid problem during biological assays (e.g. multi-plate reader measurements). As shown in Fig. 5.10, objects with 9 wells and every 3 of them were connected by a microfluidic, were printed, indeed the aim was to design wells in which technical replicates were subjected to the same medium to decrease response variability. The system included a single inlet from which the three interconnected channels had origins. Its position was chosen to allow the fulfilment of the wells with cell culture media. Importantly, the constructs presented three different outlets to avoid cross- contamination between the replicates when the extruded medium had to be analyzed. Furthermore, to facilitate plate reading and avoid signal decrease, the thickness of the bottom was 300 μm and for the optimization of the structure, a mono material plate made of PEGDA was printed. As shown in Fig. 5.10, the design of the plate underwent several modifications to avoid the asymmetrical filling of the wells during flow application. The limitation was solved by adding an initial reservoir which had reduced this side effect.

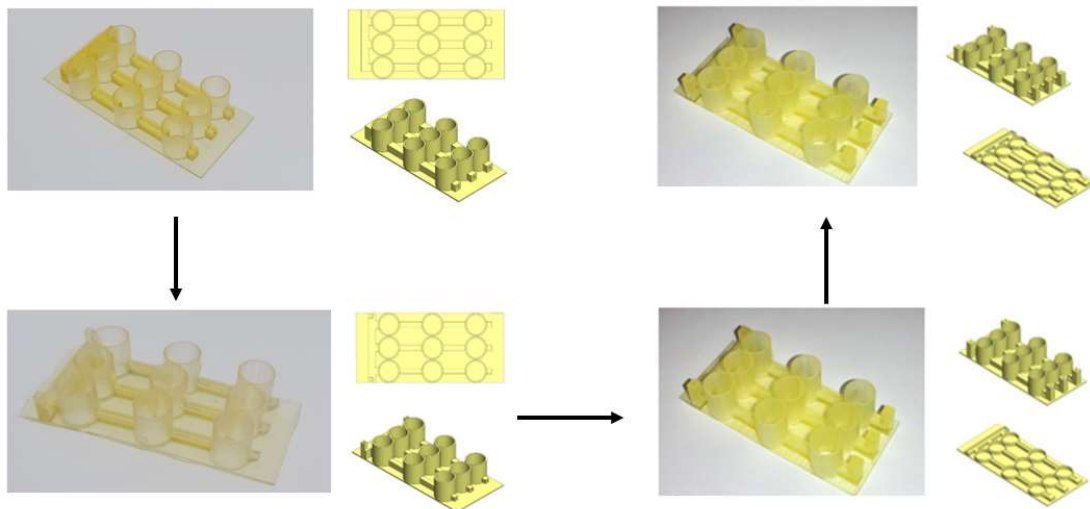


Fig. 5.10: Pictures of different printed geometries to show the implementation of the design to avoid asymmetrical filling of the printed plate.

As shown in Fig. 5.11, flow analysis demonstrated that our design was compatible with a microfluidic system. Importantly, two different channels geometry were tested: a square with a side of 1 mm (Fig. 5.11a-b) and a rectangular section whose side were 2 mm and 1 mm (Fig. 5.11c-d).

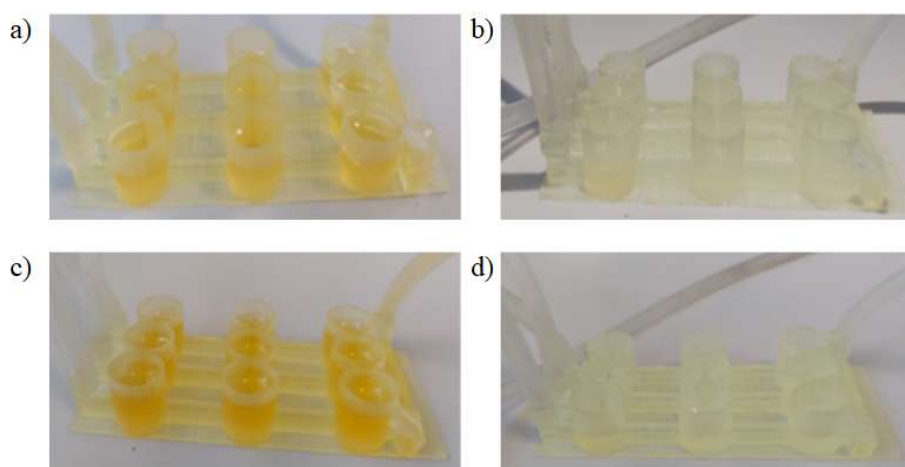


Fig. 5.11: Flow analysis in printed plate with two different geometries on the channels: a square with a side of 1 mm (a,b) and a rectangular section with 1 mm and 2 mm sides (c,d). The coloured liquid showed the complete filling of the plate (a,c) whereas transparent one the complete renewal of the liquid during the fluid flow (b,d).

Next step was the introduction of the second material, to improve microfluidic resolution and to avoid the problem of molecules retention. Further, TEGORad was chosen because of its lack of cell adhesive moieties which prevented cell adhesion on the channels of the microfluidic device. Since PEGDA allowed the adhesion of TEGORad during the printing procedure, we designed a plate in which the bottom was made in PEGDA to promote cell adhesion, whereas the upper part and the channels consisted in TEGORad. As shown in Fig. 5.12, the printing of a bi-material microfluidic plates was possible thanks to the of adding several structural elements around the wells to maximize structure integrity after printing, post-curing and washing steps.

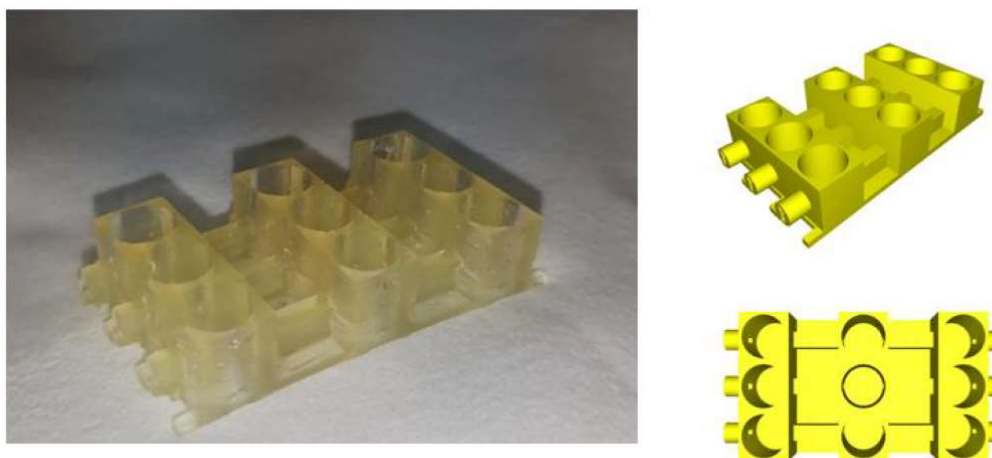


Fig. 5.12: Picture of the printed sample and the CAD of the multi-material plate.

5.4. Conclusions

In this chapter, the feasibility of using a DPL-based bioprinter to obtain microfluidic devices for cell culture and analysis was demonstrated. Specifically, three different acrylate resins, i.e. PEGDA, HDDA and BEDA, were tested, which showed physical properties compatible with 3D printing and fast photopolymerization. Post-printing analyses revealed the high presence of unreacted species which were successfully removed thanks to the development of an optimized washing protocol. In the end, PEGDA with 0,2% of BAPO results to be the formulation of election thanks to its biocompatibility, transparency and integrity post-printing. The lack of strong toxicity of TEGORad, the material used to give flexibility and to build fluidics in the device, was demonstrated. In addition, its capability to avoid hydrophobic molecules retention made it a good alternative to the commonly used PDMS. Further, its printability allowed the design of microfluidic devices, whose production could be automatized and optimized. The preliminary data obtained showed the possibility to build 3D printed microfluidic well-plate suitable for cell culture and biological assays.

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Chapter 6

Conclusions and future perspectives

Lung cancer is the main cause of cancer-related death worldwide ¹, because of its high heterogeneity and complex microenvironment ^{2,3}, which in turns leads to treatment failure and metastasis formation ^{4,5}. For many years, animal models have been used to study tumor molecular features and drug efficacy ⁶, however, differences with human and ethical concerns lead to the development of more efficient *in vitro* systems ⁷. While 2D culture fails in resembling physiological conditions ^{8,9}, 3D ones succeed in reproducing stimuli and interactions which are completely lost in the 2D models. The main goal of this project was the design of a 3D *in vitro* model able to resemble lung cancer microenvironment, to improve the process of drug development and testing and to enhance the reliability of the obtained results. In the first part of the study, the attention was focused on the synthesis and the characterization of a matrix able to support cell growth. Indeed in the tumor niche, cells are surrounded by a rich extracellular matrix, ^{10,11} which produces mechanical stimuli that modulate cells' behavior and drug response ¹². Methacrylated gelatin (GelMA) was selected as the material of election, because of the presence of cell adhesive sites ¹³ and the possibility of stiffness modulation by changing its concentration and the degree of gelatin substitution. To obtain a photo-crosslinkable methacrylated gelatin, a photoinitiator has to be added to the hydrogel formulation. So, to start the reaction of photopolymerization, a water-soluble photoinitiator, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate named LAP, was chosen, because of its activation in the visible range and biocompatibility ^{14,15}. Since many players are involved in tumor progression ⁴, the analyses were performed also in a co-culture system, in which fibroblasts (MRC-5) and tumor cells were both present. Specifically, two different adenocarcinoma models were studied, using A549 or H1299 cells, because of their mutational patterns, which could lead to different behavior and drug response ¹⁶. Further, to dissect the role of the stroma, two ratios of tumor cell and fibroblasts were assessed, i.e. 1:1 and 1:2. Systematic screening of proliferation rate in the co-culture led to the selection of two different GelMA formulations for each model, i.e. High and Very High degree of functionalization, at the concentration of 10 w/v% for A549 model and of 12.5 w/v% for H1299 one. Importantly, cell viability remained up to 80% for both models after 14 days of culture and the fibroblasts were maintained in the co-culture. During tumor progression, modification of the extracellular matrix plays an important role in the creation of a pervasive microenvironment ¹⁷⁻²⁰, thus, the expression of several proteins involved in this process was assessed. Normal fibroblasts were strongly activated when cultured in a 3D microenvironment, leading to the up regulation of α -sma, type I collagen, fibronectin and MMP2 expression. Interestingly, this modulation was completely lost when they were cultured with the tumor cells, i.e. A549 and H1299, suggesting an interplay between the two cell types, that affect fibroblasts behavior. From the literature, it is known that an active cross-talk occurs between tumor cells and the stroma during cancer growth and tumor niche definition ²¹⁻²³ and fibroblasts are usually activated to produce a fibrotic microenvironment ¹⁷. This modulation could change

according to the tumor types; thus, future analysis will be performed to understand deeply how MRC-5 behavior is modulated by A549 and H1299. Furthermore, the gene expression of TGF β , a cytokine involved in fibroblast activation and in inflammation^{24,25}, was enhanced in the co-culture after 14 days. The modulations observed were different according to the GelMA formulation used, suggesting that the matrices could have an active role in the variation of cell behavior, that in turn defined the reliability of the designed models. As well known, stiffness strongly influences gene activation, protein expression and drug response¹², thus, in the future experiments will investigate the variation in the activation of transcription factors and intracellular pathways connected to mechanotransduction, i.e. YAP/TAZ^{26,27} and Rho/ROCK^{28,29} signaling pathways.

In cancer research, another widely used *in vitro* model is based on spheroids formation^{30,31}, hence, the capability of different GelMA formulations to sustain NSCLC spheroid growth was tested. Specifically, a preliminary analysis was conducted by using A549 and, as well as in the previous analysis, the choice of the right formulation was crucial to successfully obtain bigger, transparent and healthy aggregation, specifically, High and Very High at 7,5 w/v% showed better outcomes. Reproducibility and automation could improve the reliability of our models, so, GelMA printability and the possibility to give rise to NSCLC spheroids were assessed. For this study, extrusion-based 3D bioprinting was used. This 3D printing technology, which implies pneumatic-driven dispensing, shows good results in preserving cell viability³² and it can be used to obtain functional models for cancer research^{33–35}. Printing parameters have been optimized to reduce cell death, as well as the design of efficient geometries (round, ring and grid) to allow the delivery of nutrients and oxygen and to avoid necrosis in the NSCLC spheroid formation process. The feasibility of obtaining cell aggregates after the bioprinting procedure was demonstrated, further grid geometry led to the formation of bigger spheroids, suggesting that a higher surface area exposition to the medium and consequently a higher nutrient availability, played a crucial role in the growth process. Further experiments will be performed to analyze the molecular features of those aggregates to compare them with *in vivo* tumor properties. Then, since co-culture was supported by the matrix, multi-cellular spheroids will be derived, so to investigate the crosstalk between the tumor cells and the stroma.

GelMA printability allows the possibility to build complex structures, so to develop a 3D biomimetic *in vitro* model as required for most future applications in tissue engineering and regenerative medicine^{33,34,36}. Since angiogenesis and vasculature play a crucial role in supporting tumor growth^{37,38}, the use of sacrificial inks that could be easily removed after printing, enables to obtain empty channel inside the scaffold³⁹. For that role, Carbopol polymer has been selected, because of its printability^{40,41}, and the potential use as sacrificial ink,⁴² which can be removed easily by washing⁴³, as already shown in the literature. Hence, three different Carbopol types, i.e. ETD 2020 NF, Ultrez 10 NF and NF-980 were tested to determine their mechanical properties, printability (i.e. rheological analysis and printing resolution evaluation) and cytocompatibility. The main goal was to investigate their potential use as bioinks (i.e. material loaded with cells). All of them were characterized by an elastic and shear-thinning behavior, while, after the application of a stimulus, they were able to recover the initial viscoelastic properties. Although, the lack of cell adhesive moieties, ETD 2020 and Ultrez 10 NF based formulations supported

cell viability, demonstrating biocompatibility, on the contrary NF-980, which required higher printing pressure, fails in supporting cell growth. Although Carbopol lacked long-term stability and cell-adhesive motifs, it can be successfully employed as sacrificial ink during the printing of complex scaffolds that required internal empty channels. Hence, further investigation will be conducted to test its potential to create complex empty geometries inside GelMA 3D printed scaffolds. The addition of endothelial cells in vessel-like channels will increase the reliability of the lung cancer model, because of the resembling of their role in angiogenesis and in drug absorbance.

At last, the feasibility of using a DPL-based printer to obtain a platform for cell culture and drug test analysis was investigated. Certainly, the development of microfluidic devices, which reproduce fluid flow, pressure and chemical gradients could speed up the process of drug development. Moreover, the possibility to reproduce the interaction and the paracrine signaling that occurs *in vivo*, will improve the prediction of drug effects in this complex environment, during cancer progression and treatment ⁴⁴. To select the most suitable materials to print the cell culture platform, three different acrylate resins, i.e. PEGDA, HDDA and BEDA were tested thanks to their printability and fast photopolymerization. The removal of all unreacted species was improved through the optimization of post-printing procedure (i.e. post-curing and washing steps) and the biocompatibility of the constructs was further enhanced by varying the amount of the photoinitiator (i.e. BAPO). At the end of the analysis, PEGDA containing 0,2% of BAPO results to be the optimal formulation thanks to its transparency and integrity after the printing process and biocompatibility. Hence, it was used as bulk material for a 3D printed microfluidic platform, where 3D NSCLC models could be cultured. Looking for a material for the microfluidic structure was crucial to set up a dynamic cell culture platform. To achieve that, higher printing resolution and less fragile resins were necessary, moreover, transparency and harmlessness towards cells remained essential requirements. A widely used material to create microfluidic devices is PDMS, a silicone elastomer, because of its cheapness, cytocompatibility, optical transparency, flexibility, chemical stability, and gas permeability ⁴⁵. Unfortunately, low reproducibility dependent on manufacturer, absence of printability and thermal-driven polymerization ⁴⁶ makes PDMS not suitable for our application. Hence, the study focused on an acrylate PDMS copolymer, commercially known as TEGORad, which could be 3D printed ⁴⁷. Beyond high cell compatibility and high resolution when printed, it showed lower retention of hydrophobic molecules, here used to mimic drug like molecules, than PDMS, demonstrating TEGORad potential as building material for microfluidic. Further studies suggested the feasibility to obtain a 3D multi-material well-plate whose wells, made of PEGDA 0,2% BAPO, were connected to each other by acrylate PDMS-based empty channel allowing a dynamic cell culture system.

In the future, NSCLC models developed and study during this PhD thesis will be cultured in 3D printed microfluidic multi wells to assess the effects of dynamic culture conditions (i.e., continuous medium flow) on cell behavior. Further, spheroids embedded in GelMA will be directly printed inside the wells to completely automatize the process of cell culture preparation. In parallel, computational analysis will be performed to evaluate the formation of hypoxic region inside the scaffold to optimize the geometries of 3D *in vitro* internal channels.

To conclude, in this thesis a novel *in vitro* system to study lung adenocarcinoma properties has been described. Specifically, the introduction of a 3D environment to mimic tumor niche and the possibility to print this model to decrease manufacturer's variability, increased the reliability of the model. Further, the designing of a microfluidic platform allowed to obtain perfused chambers to better mimic the fluid exchange that was usually present in physiological condition. Overall, these implementations may contribute to reach 3R principles, hence the goal of reducing *in vivo* models for the analysis of tumor features and drug response.

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Appendix I

List of publications

- (1) Nicolò, C.; Parmeggiani, M.; Villata, S.; **Baruffaldi, D.**; Marasso, S. L.; Canavese, G.; Cocuzza, M.; Pirri, C. F.; Frascella, F. A Programmable Culture Platform for Hydrostatic Stimulation and in Situ pH Sensing of Lung Cancer Cells with Organic Electrochemical Transistors. *Micro Nano Eng.* **2022**, *16* (March). <https://doi.org/10.1016/j.mne.2022.100147>.
- (2) Gonzalez, G.; **Baruffaldi, D.**; Martinengo, C.; Angelini, A.; Chiappone, A.; Roppolo, I.; Pirri, F. C.; Frascella, F. Materials Testing for the Development of Biocompatible Devices through Vat-Polymerization 3D Printing. *Nanmaterials* **2020**, *10* (9), 1788. <https://doi.org/doi:10.3390/nano10091788>.
- (3) Zanon, M.; **Baruffaldi, D.**; Sangermano, M.; Fabrizio Pirri, C.; Frascella, F.; Chiappone, A. Visible Light-Induced Crosslinking of Unmodified Gelatin with PEGDA for DLP-3D Printable Hydrogels. *Eur. Polym. J.* **2021**, *160* (August), 110813. <https://doi.org/10.1016/j.eurpolymj.2021.110813>.
- (4) **Baruffaldi, D.**; Palmara, G.; Pirri, C.; Frascella, F. 3D Cell Culture: Recent Development in Materials with Tunable Stiffness. *ACS Appl. Bio Mater.* **2021**, *4* (3), 2233–2250. <https://doi.org/10.1021/acsabm.0c01472>.
- (5) **Baruffaldi, D.**; Pirri, C. F.; Frascella, F. 3D Bioprinting of Cell-Laden Carbopol Bioinks. *Bioprinting* **2021**, *22* (February), 1–5. <https://doi.org/10.1016/j.bprint.2021.e00135>.
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- (7) Coppo, R.; Orso, F.; Virga, F.; Dalmasso, A.; **Baruffaldi, D.**; Nie, L.; Clapero, F.; Dettori, D.; Quirico, L.; Grassi, E.; Defilippi, P.; Provero, P.; Valdembri, D.; Serini, G.; Sadeghi, M. M.; Mazzone, M.; Taverna, D. ESDN Inhibits Melanoma Progression by Blocking E-Selectin Expression in Endothelial Cells via STAT3. *Cancer Lett.* **2021**, *510*, 13–23. <https://doi.org/10.1016/j.canlet.2021.04.005>.
- (8) Dettori, D.; Orso, F.; Penna, E.; **Baruffaldi, D.**; Brundu, S.; Maione, F.; Turco, E.; Giraudo, E.; Taverna, D. Therapeutic Silencing of MiR-214 Inhibits Tumor Progression in Multiple Mouse Models. *Mol. Ther.* **2018**, *26* (8), 2008–2018. <https://doi.org/10.1016/j.ymthe.2018.05.020>.

Appendix II

International conferences and schools

European Chapter 2023 TERMIS-EU Conference

Manchester – 28th – 31st March 2023

Podium presentation: **Baruffaldi D.**, *Canta M., Villata S., Monica V., Pirri C.F., Frascella F.* “3D-printed cell culture system as an in vitro platform for non-small cell lung cancer (NSCLC) modeling”

NanoInnovation 2022 Conference&Exhibition

Roma – 19th – 23rd September 2022

Invited: **Baruffaldi D.** “Three-dimensional cell culture system as an in-vitro platform for lung cancer modelling”

European Chapter 2022 TERMIS-EU Conference

Cracovia - 28th June -1st July 2022

Poster: **Baruffaldi D.**, *Canta M., Monica V., Pirri C.F., Frascella F.* "Three-dimensional cell culture system as an in vitro platform for lung cancer modeling"

III Convegno Annuale del Centro 3R "L'era delle 3R: modelli in silico, in vitro e in vivo per promuovere la ricerca traslazionale

Online - 30th September – 1st October 2021

Poster: **Baruffaldi D.**, *Canta M., Pirri C.F., Frascella F.* "Development of in vitro 3D culture system to mimic lung cancer tissue"

26th Congress of the European Society of Biomechanics

Online – 11th -14th July 2021

Podium presentation: **Baruffaldi D.**, *Pirri C.F., Frascella F.*, "3D printing of microfluidic devices for cell culture"

Bioprinting Winter school 2020

Pavia - 11th -14th February 2020

Poster: **Baruffaldi D.**, *Martinengo C., Frascella F., Pirri C.F.* "Gelatin methacrylamide GelMA mechanical characterization and printing"