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Study of the interaction between nanoparticles and cancer cells in unicellular 3D models

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Wit beyond measure is man's greatest treasure

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Abstract

Cancer is the second leading cause of death in industrialized countries. Tumors affect different organs and tissues; they are characterized by uncontrolled cells proliferation which impair function and structure of involved body area. The high mortality rate of cancer is due to the generation of metastases, which seems to take place only in specific organs, mainly the liver, lung and bones.

Mechanical properties of extracellular matrix are known to support cell survival and spreading and might therefore play a role in the metastatic spreading of cancer.

Here, we aim to prepare different substrates of similar fibrous morphology but different mechanical properties that mimic those of the organs which are most affected by metastasis: liver, bone and lung. Modulation of the mechanical properties was achieved by proper selection of the polymer constituent of the fibers, namely poly(ϵ -caprolactone) (PCL), gelatin (GEL), a blend of PCL and GEL and the same blend doped with nanoscale hydroxyapatite. Nanofibrous membranes were prepared by electrospinning technique and characterized in terms of morphology and mechanical properties. Cells derived from primary pancreatic tumors were seeded on the constructs and their colonization, morphology, and viability were assessed overtime. Hybrid polymer/lipid nanoparticles were then designed and characterized and their behavior in culture with cells seeded on the different constructs was examined to evaluate whether the interaction between therapeutic carriers and target cells may depend on the mechanical properties of the tissue. Our preliminary results suggest that the choice of the construct material affects the viability of seeded cells, regardless of the cell line used. Moreover, we showed that the capacity of cells to internalize nanoparticles is also affected by the substrate on which they are grown. Future works should focus on expanding our tests on cells derived from different primary tumors to confirm our findings.

Chapter 1 – Introduction

1.1 – Cancer

Cancer is the second leading cause of death worldwide, only preceded by cardiovascular diseases in industrialized countries. It was estimated that 9,6 million of people died of cancer in 2018 [1] and the number of new cases has continued to increase in the last twenty years at a constant rate [2].

Tumors are a group of diseases that affect different organs and tissues; characterized by abnormal cell proliferation.

Cancer can be considered as a genetic disease caused by variations in genes, inherited or arisen during lifetime due to errors in cell division produced by external factors. These environmental agents can be physical, like ionizing radiations or UV, chemical, such as carcinogen substances contained in food, tobacco, alcohol, or biological, like infections [1].

In the advanced stage, malignant tumors develop the ability of spread to other parts of the body in a process called metastasis; this does not happen in benignant tumors that lack the capacity to invade bordering tissues or metastasize. The mortality rate of the metastatic disease is 90% [3].

The metastatic process, schematized in **Figure 1**, begins when circulating tumor cells leave the primary site and through the penetration into lymphatic or circulatory system, called respectively lymphatic and hematogenous spread, reach other body areas creating a secondary tumor [4].



Fig 1. Diagram of metastatic process.

The organs or tissues in which metastases spread depend on the characteristics of primary tumor, but some sites are common to most cancer types. According to the work of Hess and collaborators [5], the main target organs are liver and lung, because of their large vascularization, and bones. The diagram shown in **Figure 2** highlights organs most affected by different types of tumor. Except for lymph nodes which invasion states the first step of metastatic process, a great percentage of patients presents metastases to the bones and to the lung, for almost all type of tumors analyzed. Among the patients affected by colon cancer and pancreatic cancer around 80% develops metastases to the liver and, with a lower percentage, the patients which primary tumor was in the lung, breast, or kidney.



Fig 2. Percentage of patients with metastatic sites for each primary tumor.

The most common tumors are breast cancer, lung cancer, prostate cancer, colorectal cancer, pancreatic cancer and liver cancer [6].

These tissues present different mechanical properties, which are mainly dependent on the composition of the extracellular matrix (ECM), that is tissue specific (as shown in **Figure 3**).

Since all human cells, excluding blood cells, live in the complex three-dimensional network of the ECM, the tendency of certain cancer cells to metastasize only to specific tissues and organs may be dependent on the mechanical properties of the ECM of these target organs.

Indeed, the ECM has different mechanical properties in different tissues, contributing significantly to the stiffness or elasticity of the organ.

Mechanical properties of tissues influence numerous cell processes, such as migration, proliferation, differentiation and apoptosis. It has been shown that cells are responsive to external forces, whether they are originated from ECM, from a synthetic subtract or from an adjacent cell [7].



Fig 3. Structure and components of ECM.

Stiffness and elasticity of tissues can change during lifetime due to ageing or disease. A common pattern emerges from the study of tumors in which the stiffening of tissues increases with disease progression [8]. Cancer cells show higher viability and rate of proliferation on more rigid substrates compared to the ECM of the corresponding normal tissue, proving the trend of tumor cells to the migration toward harder tissues [9].

In this work we focused on the three main metastases-hosting organs: lung, liver and bone. The properties and composition of the ECM of these organs are described in the following paragraphs. In particular, we focused on the variation of the Young Modulus between normal and pathological tissue, shown in **Table 1**.

The Young's Modulus is several orders of magnitude higher for bone, since it is the hardest tissue. Liver and lung present comparable values and both organs exhibit an increase of elastic modulus in case of pathology.

1.1.1 Properties of the lung ECM

Lung ECM is arranged into two structures: basement membranes, composed by glycoproteins, endothelia surrounded by muscles and fat, and interstitial matrices, that interconnects cells and sustains the 3D architecture of the tissue [9].

The main proteins of pulmonary ECM are: collagen, which, with its tensile strength, maintains the structure, and elastin and fibrillin, responsible for providing compliance and elastic recoil. [10]

In lung diseases an increase of ECM stiffness occurs. For example, in idiopathic pulmonary fibrosis (IPF) the fibroblasts deposit high levels of ECM molecules which lead to an intensification of tissue rigidity [11]. In cancer, both primary and metastatic, cells are surrounded by a stiff stroma containing highly crosslinked collagen and high levels of fibronectin [12].

The remodeling of pathological extracellular matrix leads to its stiffening causes the formation of a mechanical gradient between healthy and abnormal tissue which, in turn, promotes cells migration. In fact, the migratory behavior of cancer cells follows rigidity variation [13].

1.1.2 Properties of the liver ECM

The main components of liver ECM are glycoconjugates and proteins, such as collagen and fibronectin, which are distributed mainly in the Glisson's capsule, portal tracts and in the ducts of the biliary tree [14]. The basement membrane composed by laminin, fibronectin and proteoglycans is found in the space of Disse.

In pathological liver an increase of stiffness is verified due to an overproduction of ECM, for example in cirrhosis there is an increase of glycoproteins which results in up to a six-fold increase in ECM production than in the normal organ [14].

1.1.3 Properties of the bone ECM

Bone is a connective tissue composed by cells and extracellular matrix that consists of a natural organic component and an inorganic part, at a ratio of 30:70. The organic component is characterized for 90-95% by collagen fibers which regulate the tensile strength and for the remaining part by proteoglycans and other proteins. The inorganic mineral component, which provides support, is constituted by salts of calcium and phosphate, mainly hydroxyapatite, and controls compressive strength. Collagen fibers trap hydroxyapatite crystals creating orderly bundles that overlap one another giving the bone its total strength [15].

According to the collagen placement, two types of bones are recognized:

- 1) cortical, or woven, that is weaker due to randomly oriented collagen fibers;
- 2) trabecular, or lamellar, mechanically stronger because of organization of collagen into regular parallel sheets (lamellae).

Organ	Young's modulus	Reference
LUNG		
Healthy	1,96 ± 0,13 kPa	[10]
Pathological (IPF)	16,52 ± 2,25 kPa	
LIVER		
Healthy	3-6 kPa	[16]
Pathological (fibrosis, cirrhosis)	20 kPa	
CORTICAL BONE TRABECULAR BONE	7,4-31,6 GPa 6,9-23,5 GPa	[17]

Table 1. Young's moduli of the healthy and pathological tissue for studied organs.

1.2 – The tissue engineering approaches

Tissue engineering (TE) is the study of the repair, replacement and regeneration of damaged organs or tissues. TE may use three main approaches: use of isolated cells, decellularized scaffolds or cells-seeded scaffolds [18] [19].

In this work, we choose to cultivate cells on natural and synthetic substrates to realize a 3D biological substitutes with functional properties and architecture that mimic those of specific organs extracellular matrix. The TE approach is based on three components:

- Scaffold → 3D porous matrix that allows cell adhesion, growth and proliferation. It is constituted by nanofibrous fibers synthetized through electrospinning, self-assembly or phase separation techniques [20].
- Cells \rightarrow on the base of realizing tissue the choice can fall on:
 - ✓ primary cells, removed from a patient, represent the functional units of the tissue, but they have a complex process of isolation, limited life span and slow proliferation;
 - ✓ cell lines, immortal differentiated cells removed from tumors or primary cells modified to proliferate indefinitely;
 - ✓ stem cells, undifferentiated cells which are capable of long-term self-renewal and of differentiating to fully specialized cells. [21]
- Regulators \rightarrow static or dynamic culture, administration of mechanical and biochemical stimuli.

The approach selected for this study is an *in vitro* static culture of cancer cell lines seeded on scaffolds.

One fundamental step in scaffold realization is the selection of the proper biomaterial, which affects cellular behavior. Moreover, the material must provide mechanical support for cell adhesion and survival.

The choice of material is made in accordance to the target organ of interest, considering mechanical properties of healthy and pathological tissues.

Among the different materials used in tissue engineering, polymers are the most interesting for their advantages, such as high porosity, controllable pore size, chemical versatility, high surface/volume ratio, good mechanical properties [22] [23].

Synthetic materials, such as poly- ε -caprolactone (PCL), polylactic acid (PLA), polyglycolic acid (PGA), poly(lactide-co-glycolide) (PLGA), are widely used in tissue engineering. These polymers are thermoplastic, easy to process in 3D micro-structures with different shapes and they have a higher stability because of their less sensibility to temperature as compared to natural polymers. Process parameters and degradation rate can be controlled, thus improving the mechanical properties. Despite the good biocompatibility, synthetic polymers may have low bioactivity, resulting in poor cell adhesion due to their hydrophobicity [24].

Natural polymers, such as gelatin, have a high bioactivity, supporting cell adhesion and grow, and biodegradability, allowing the substitution of natural scaffold with extracellular matrix produced by cells. They have poor mechanical properties and need to be crosslinked. Natural polymers are made up of proteins and polysaccharides [25].

	Advantages	Disadvantages
Synthetic polymers	 easy to process strength stability controlled degradation good mechanical properties reproducibility 	low bioactivitypossible toxicity
Natural polymers	elevated bioactivitycontrolled degradationnontoxic	 poor mechanical properties possible immune response low reproducibility lack of stability

In Table 2 polymers characteristics are summarized according to their origin.

Table 2. Synthetic versus natural polymers.

The idea of mixing synthetic and natural polymers comes from the necessity of optimizing the advantages and reducing the disadvantages of the two categories. In fact, a polymer with appropriated mechanical properties and stability that, also, has a good interaction with cells can efficiently mimic tissues ECM [26].

In this work, a synthetic polymer (PCL), a natural polymer (gelatin, GEL) and a mixture of both were tested to evaluate the influence of their mechanical characteristics on the interaction with cells. Moreover, an additional material composition was experimented by adding nanohydroxyapatite to PCL/GEL blend to increase the mechanical strength and better imitate the bone tissue ECM.

1.3 – Aim of the work

This thesis work intends to evaluate how mechanical properties of different substrates influenced the growth and proliferation of cancer.

To achieve this aim, we synthesized different substrates with similar nanofibrous aspect, but with different mechanical properties – modulated through the choice of different materials. In particular, four different compositions were tested, namely:

- 1. Synthetic poly(ε-caprolactone) (PCL);
- 2. Cross-linked gelatin;
- 3. A PCL/Gelatin blend at the ratio of 80:20;
- 4. A PCL/Gelation ration containing nano-scale hydroxyapatite.

The mechanical properties are intended to mimic those of ECM of organs that are frequently affected by metastases, that are lung, liver and bone.

Cells receive signals from other cells and from the ECM, so it is essential that 3D porous scaffolds mimic the structure and the functions of specific tissue ECM and supports cells in their adhesion, proliferation and migration [27]. To achieve this, the scaffold chemical composition must be similar to that of the native tissue and the architecture must provide the proper size and porosity to facilitate cell colonization. Moreover, the mechanical properties are fundamental, the scaffold provides stability without interfering with cell adhesion or migration. In this work we designed nanofibrous supports with random fiber orientation that closely mimic the fibrillar structure of the ECM.

After a complete morphological and chemical characterization of hydroxyapatite, nanofibers were realized from the above-mentioned polymer solutions by the electrospinning technique and their morphology and mechanical properties were evaluated.

As a model of primary tumor, we focused on two pancreatic cell lines, MIA PaCa and PANC-1. We focus on pancreatic cancer cells as a model of primary tumor.

Pancreatic cancer is known to metastasizes, mainly, to the liver and the lung, as represented in **Figure 4**, with little bone involvement.



Fig 4. Metastatic sites caused by pancreatic cancer [5].

Two pancreatic cancer cell lines were seeded on the substrates and morphology, viability and hybrid polymer/lipid nanoparticles internalization were assessed.

Cells were cultured on the different substrates and the viability and morphology were monitored at two different time-points. We, then, tested whether cells cultured on different substrates respond differently to treatment with polymer/lipid nanoparticles. In particular, we measured cell internalization on cells cultured on the substrates, as compared to cells in 2D culture.

We showed that the mechanical properties of the PCL/GEL blend doped with hydroxyapatite, which presented high resistance, positively influenced the viability of cells cultivated on this substrate, as well as the internalization of nanoparticles. Even if gelatin nanofibers appeared the most suitable substrate to cultivate the cell lines tested, the PCL/GEL/nHA blend also demonstrated to be an interesting alternative for further studies.

1.4 – The electrospinning technique

Tissue engineering is based on the creation of micro-nanoscale substrates that constitutes a suitable environment for cells survival.

Electrospinning is an efficient, rapid and relative low-cost fiber production method. It allows to obtain nanofibrous scaffolds, made by synthetic and natural polymers with a high porosity, that mimic tissues extracellular matrix. Electrospinning technique uses an electrical field to control fibers deposition on a collector.

The diameter of obtained fibers is in the range of micron up to 2 nm, the same of ECM proteins. Fibers dimension and membrane structure have a relevant influence on cell adhesion and proliferation, in fact nanofibers present a high surface to volume ratio and a size significantly lower than cells which guarantee a 3D cell grow and morphology like the *in vivo* one [28].

1.4.1 Electrospinning instrumentation

The instrument utilized in this study is Electrospinning Linari Engineering (pictured in **Figure 6**) and its components, schematized in **Figure 5** are:



Fig 5. Schematic diagram of electrospinning.

- Syringe: according to the type of solvent utilized can be made of glass or plastic, has a volume of 5 ml and a needle of 0,8 mm (21 G).
- Volumetric pump: exerts a continuous pressure on the syringe plunger, the solution is extruded at an imposed constant speed (Linari Engineering s.r.l).
- High voltage source: generates voltage in the range of 0-60 kV (Linari Engineering s.r.l).

- Static collector: composed by a plexiglass flat plate coated with an aluminum sheet which collects random nanofibers.
- Glass circular coverslips: with a diameter of 12 or 22 mm, collect random nanofibers. They are fixed upon the aluminum sheet with conductive double-sided adhesive tape.
- Vented hood: sets the ventilation to aspirate solvent vapours and the temperature.

Electrospinning is based on a huge electric field that acts on a polymer solution contained in the syringe and extruded thanks to the pressure generated by the volumetric pump. The high potential difference applied speeds up the solution against the collector. Random nanofibers produced by solvent evaporation deposit on the collector creating a 3D structure.

Several variables influence the electrospinning process, so the regulation of parameters is fundamental to obtain specific fibers dimension and morphology. [29]

Electrospinning parameters concern the instruments utilized during the process and they are:

- Electrical field → at critical voltage, which value depends on the polymer, the spherical droplet
 of solution deforms into a Taylor cone and generates nanofibers. Higher voltage causes
 smaller-diameter fibers due to the stretching of polymer solution together with the charge
 repulsion, but too high-tension values can increase fibers size.
- Distance needle-collector → allows solvent evaporation; the diameter of fibers decreases with the increase of distance.
- Flow rate → concerns jet velocity and solvent evaporation; a low flow rate permits a complete solvent evaporation and the formation of smaller diameter fibers.

Solution parameters depend on polymer solution properties, they are:

- Solvent → has influence on matrix porosity. Volatile solvents are used because their high evaporation rate increases pores density.
- Polymer concentration → is related to solution viscosity and influences fibers formation. In the range of values suitable for electrospinning, below minimum concentration the electrospray process substitutes the electrospinning one and above maximum concentration the viscosity is too high to consent the extrusion of solution, increasing the concentration fibers size grows.
- Solution viscosity → is related to polymer concentration and molecular mass of the components. An increase of molecular mass entails larger diameter fibers.
- Solution conductivity → is related to solution composition, almost all polymers are conductive.
 Increasing conductivity, fibers diameter lowers.

Lastly, environmental parameters are:

- Humidity \rightarrow influences matrix porosity acting on solvent evaporation.
- Temperature → an increase of temperature causes a quickly solvent evaporation and a higher viscosity with smaller diameter fibers.

1.5 – Nanoparticles

Nanoparticles (NPs) are nano-sized particles with a diameter in the range of nanometers. They are widely studied in biomedical field as a carrier for drugs in drug delivery and in cancer therapies [30]. Nanoparticles have the ability to encapsulate drugs inside their matrix or absorb them on the surface. This allows NPs to carry active substances directly to the tumor site protecting them from the degradation caused by body environment [31]. The nano dimension enhances the penetration potential inside tissues and the uptake by cancer cells through passive targeting mechanism. The abnormalities of the tumor vascular system, characterized by the presence of fenestrations, and the lack of a lymphatic system increase the absorption and the retention of small particles in tumors compared to normal counterparts. This property of cancer is known as Enhanced Permeability and Retention (EPR) effect [32]. Other methods to target NPs to the desired organ include the application of ultrasounds or magnetic field or the surface functionalization, favored by high surface/volume ratio, with proteins that interact with cells.

Nanoparticles used for medical application are composed of polymers or lipids because their intrinsic biocompatibility.

Polymer nanoparticles present structural stability and better mechanical properties. Moreover, they can be designed to achieve controlled and tunable drug release profiles [33].

Lipid based nanoparticles, such as liposomes, can efficiently deliver hydrophilic and hydrophobic drugs with a low toxicity, they have aqueous stability and controlled release properties [34]. Drawbacks of these NPs depend on the lack of structural integrity.

Hybrid lipid/polymer nanoparticles are synthetized to increase the advantages of polymer and lipids as NPs components. Two materials assemble in a core-shell system, schematized in **Figure 6**: the core is made by the polymer that stabilize the particle structure and encapsulate the drug whiting the matrix; the shell is coated by lipids which guarantee stability in aqueous environment (thanks to the phospholipids behavior) and protect the central part from degradation. An outer lipid-PEG layer is added with antifouling function, to protract NPs circulating time and avoid immune system response [35].



Fig 7. Lipid/polymer core-shell structure.

1.5.1 Nanoparticles preparation methods

The most used nanoparticles synthesis techniques are nanoprecipitation and solvent evaporation [36].

Nanoprecipitation method, represented in **Figure 7**, establish the polymer dissolution in a water miscible organic solvent, while lipid and lipid-PEG are dissolved in water providing heat to avoid micelle formation. When the polymer is dropped into lipidic dispersion, it coils into the center of NPs and the phospholipids arrange themselves with the hydrophilic heads toward the external environment and the hydrophobic tails directed to the polymer core. Lipid-PEG has the same behavior: hydrophobic lipidic tails point to NP shell and PEG chain external. This method allows hydrophobic drug encapsulation in the polymer core.



Fig 7. Nanoprecipitation technique for NPs preparation.

Solvent evaporation requires the dissolution of polymer in a volatile organic solvent and an aqueous phase containing lipids. Mixing the two preparations, nanoparticles are obtained by solvent evaporation, because of magnetic stirring or temperature increasing, followed by centrifugation.

A variation of this method, called emulsification, allows drugs encapsulation. For hydrophobic drugs is used a single emulsion technique, schematized in **Figure 8A**, or oil in water (o/w), that implicate the formation of an emulsion between drug and polymer dissolved in water immiscible oil phase and lipids in aqueous phase. Drug and polymer concentrate in the hydrophobic core surrounded by lipidic shell. Double emulsification, shown in **Figure 8B**, or water in oil in water (w/o/w) permits to carry two substances or drugs, one hydrophilic in the aqueous core surrounded by a fist lipidic shell and one hydrophobic in the polymer layer with, eventually, different release kinetics. An outer lipid-PEG shell envelops the NP.



Fig 8. Single emulsion o/w (A) and double emulsion w/o/w (B) technique for NPs preparation.

In this work hybrid nanoparticles were prepared by nanoprecipitation of a polyurethane into two lipids mixture and, then, labelled with a fluorophore.

Chapter 2 – Materials and methods

2.1 – Materials

2.1.1 Polymers

The polymers used in this study are $poly(\epsilon$ -caprolactone) (PCL) and gelatin.

2.1.1.1 PCL

PCL (chemical formula shown in **Figure 9**) is a synthetic, semi-crystalline, biodegradable polymer with an elevated biocompatibility due to its high solubility in organic solvents and low melting temperature, around 58-60°C [37]. PCL is easy to process and degrades by hydrolysis in 2-3 years [38]. These features, coupled with the excellent biocompatibility, good mechanical properties and facility to blend with other polymers, warranted its use in the biomedical field as scaffolding material for tissue engineering [39].



Fig 9. PCL formula [40].

PCL used in this work was purchased from Sigma-Aldrich in form of pellet and possess the characteristics summarized in **Table 3**.

Melting temperature	58-60 °C
Glass transition temperature (Tg)	-60 °C
Molecular mass	70000-90000 Da
Young's modulus	343,9-363,4 MPa

Tab 3. Properties of PCL [41].

2.1.1.2 Gelatin

Gelatin (structure shown in **Figure 10**) is a natural polymer derived from the denaturation of collagen, the main structural protein of the extracellular matrix (ECM) in connective tissues [42]. Commercial gelatin is usually obtained from mammal tissues, such as porcine skin or bovine bones, or, recently, from marine organism. It is composed by 90% of proteins, 1-2% minerals and water.



Fig 10. Chemical structure of gelatin [43].

This biopolymer in widely used in regenerative medicine and tissue engineering because of its biocompatibility, biodegradability and bioactivity which facilitate cells adhesion and growth. The relative low cost and the ease of processing contribute to the wide application of gelatin in many industrial branches. On the downside, the water solubility, the gel-sol transition at body temperature and the poor mechanical properties accentuate the importance of chemical cross-linking of gelatin [44].

Gelatin used in this work was purchased from Sigma-Aldrich in the form of powder and its properties are displayed in **Table 4.**

Origin	Pork skin
Туре	A
Water solubility	50 mg/ml
Gelation temperature	25 °C
Young's modulus	40-60 MPa

Tab 4. Properties of gelatin [45]

2.1.1.3 γ-glicidossipropiltrimetossisilano (GPTMS)

GPTMS (formula shown in **Figure 11**) is used for the cross-linking of gelatin to increase the stability of biocompatible membranes in the aqueous environment [46].



Fig 11. Chemical configuration of GPTMS [47].

The GPTMS used in this work was purchased from Sigma-Aldrich in form of liquid with 1,07 g/ml density at 25°C.

2.1.2 Hydroxyapatite

Hydroxyapatite (HA) is a mineral form of calcium apatite; the formula is $Ca_{10}(PO_4)_6(OH)_2$ and the crystal structure is reported in **Figure 12**. HA is one of the main components (65-70%) of human bones and teeth (70-80%) [48].



Fig 12. Crystal structure of hydroxyapatite [49].

Hydroxyapatite is widely used as medical ceramic in bone grafts, dental repair and prosthetics due to its properties of osteoconductivity and biocompatibility. Moreover, the bioinspired nanoscale hydroxyapatite (nHA) is able to stimulate bone tissue regeneration.

2.1.3 Cells

In this work two human pancreatic cancer cell lines were used: PANC-1 and MIA PaCa.

2.1.3.1 PANC-1

The cell line is originated from ductal cells of a 56-year-old male. These cells have the ability of create metastasis and take 52 hours to double in population. PANC-1 grow as adherent cells with tendency to clump [50].

2.1.3.2 MIA PaCa

The cell line was isolated in a 65-year-old male and has been widely used in the study of carcinogenesis in pancreas and in testing of therapeutics. *In vitro,* MIA PaCa grow both as adherent and floating cells [51].

2.1.4 Nanoparticles

The nanoparticles used in this study possess a core-shell structure [52]. The shell is composed by two phospholipids, $L-\alpha$ -phosphatidylglycerol (EGG-PG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG), while the core is made of a PCL-based polyurethane (PU100).

DSPE-PEG lipid is a PEG derivative containing lipid molecules DSPE, which improve circulation times for liposome-encapsulated drugs. Lipids used to prepare nanoparticles were all purchased from Avanti Polar Lipids.

2.2 – Methods

2.2.1 Synthesis of bioinspired nanoscale hydroxyapatite

The preparation of bioinspired nanoscale hydroxyapatite followed the protocol by Gentile and collaborators [53], which is schematized in **Figure 13**.

Calcium and phosphorus solutions were prepared adding 3,705 g of calcium hydroxide $Ca(OH)_2$ to 500 ml deionized water under stirring on a magnetic stirrer for 1 hour at 400 rpm. Meanwhile, 3,459 g of phosphoric acid (85%) H₃PO₄ were dissolved in 250 ml deionized water. The H₃PO₄ solution was poured into the Ca(OH)₂ suspension under stirring, covered maintained under stirring for 1 hour at 400 rpm. The suspension was, then, left to settle overnight. The day after, the suspension was washed by

pouring off the supernatant, followed by the addition of 500 ml of clean deionized water and stirring for 1 hour at 400 rpm. This washing step was repeated three times with 2-hour intervals. The clean nHA suspension was left settle overnight, collected and allowed to fully dry in oven at 80 °C for three days. The dried nHA was, then, placed into an inert mortar and ground into a fine powder. The nHA powder was placed in an alumina crucible, sintered at 1000 °C for 2 hours with ramp rate of 10 °C/min and left to cool in the furnace.



Fig 13. Synthesis of 5 g of nanoscale hydroxyapatite.

The process was repeated two times to verify the repeatability of synthesis.

2.2.2 Characterization of bioinspired nanoscale hydroxyapatite

2.2.2.1 Field Emission Scanning Electron Microscope (FE-SEM)

Field emission scanning electron microscopy is a non-destructive technique used to analyze the morphology of surfaces by scanning the sample with a focused electron beam. The technique, also, allows to evaluate the elements composing the material.

In a regular scanning electron microscopy (SEM), the resolving power is inversely proportional to the wavelength of the radiation, therefore the use of electrons instead of photons, with their lower wavelength, allows a higher resolution of 3D images with an elevated magnification. In addition, the FE-SEM technology guarantees high quality images due to a clearer signal together with a higher spatial resolution. [54]

As schematized in Figure 14, the microscope is composed by:

- Electron gun;
- Lens;
- Detectors;
- Sample chamber.



Fig 14. Schematic diagram of FE-SEM.

The electron beam is emitted (thermionic emission) from an electron gun fitted with a tungsten filament cathode. The emission of FE-SEM gun is reached by placing the filament in a huge electrical

potential gradient, instead of heating the metallic filament, as in the SEM gun. Through the application of a potential difference, the electrons obtain an energy in the keV range and are sped up against the anode. The beam is focused by one or two condenser lenses to a small area, with a diameter in the range of nm, than passes through a pair of scanning coils and, at the end of the column, the beam is focused on the sample by the final lens. The collision between the electron beam and the sample produce backscattered electrons, X-rays and secondary electrons, as shown in **Figure 15**.



Fig 15. Sample-beam interaction with a FE-SEM.

Backscattered electrons have an elevated energy (in the range of keV) and their intensity is related to the atomic number Z of the elements in the sample. The observation of this radiation provides a qualitative analysis of chemical elements composing the sample, even if there is a loss of resolving power.

Secondary electrons have a lower energy (in the range of 10 eV) and, when detected, are converted into electrical signals with an intensity that depends on sample's morphology. The 3D image of the superficial topography obtained has a high resolution.

X-rays are emitted from the sample after the ionization of its atoms. This allows a chemical analysis which leads to understand the elemental composition.

The whole process of image acquisition occurs in the condition of high vacuum. [55]

In this work, FE-SEM was used to evaluate the morphology of the nanoscale hydroxyapatite crystals. A small amount of nHA powder was dispersed in water and dropped on an aluminum stub, covered with a conductive double-sided adhesive tape. After drying, the stub was sputter coated with platinum (using an Agar Auto Sputter Coater) and left under vacuum overnight.

The microscope utilized is a ZEISS Supra 40 Field Emission Scanning Electron Microscopy and the images were captured at different magnifications (1000X, 5000X, 10000X, 30000X, 150000X) whit a voltage of 5 KV.

Two FE-SEM images, one for each repetition of HA synthesis process, were analyzed through the software ImageJ to measure the diameter of 30 nanoparticles of HA.

Elemental analysis and chemical characterization of nHA was performed by energy-dispersive X-ray spectroscopy (EDX) [56].

2.2.2.2. X-Ray Diffraction Analysis (XRD)

X-Ray Diffraction is a non-destructive technique used to analyze the structure of crystalline materials: it can be employed to identify the crystalline phases present in a material and, therefore, the chemical composition.

In **Figure 16** the working principles of a diffractometer are displayed: an X-ray beam, generated by a stationary source, strikes the sample, in a powder form, which rotates together with one detector [57].



Fig 16. XRD functioning scheme.

The obtained graphic, called diffractogram, has a certain number of peaks and is compared with the diffractograms contained in the database Powder Diffraction File present in International Centre for Diffraction Data (ICDD). Comparing position and intensity of each peak of the sample, the unknown compound can be identified along with the crystalline phases existing in the sample.

The nanoscale hydroxyapatite was analyzed by XRD in sintered and non-sintered form. The acquired data of both samples is compared with the diffractogram of hydroxyapatite available in the database.

2.2.3 Polymer solutions for electrospinning

Following optimized protocols provided in the literature, four polymer solutions of PCL, gelatin, PCL/gelatin and PCL/gelatin/nanohydroxyapatite were prepared as schematized in **Figures 17-18-19-20** for further processing by electrospinning technique.

2.2.3.1 Preparation of the PCL solution

PCL solution (5 ml) was obtained by dissolving the polymer (12% w/v) in a 70:30 mixture of chloroform (CHCl₃) and formic acid (CH₂O₂), followed by 1 h stirring at 400 rpm.



Fig 17. Schematic diagram of PCL solution preparation.

2.2.3.2 Preparation of the Gelatin (GEL) solution

Gelatin solution (3 ml) was obtained by dissolving the polymer (15% w/v) in deionized water, followed by 1 h stirring at 400 rpm. Then, GPTMS (92 μ l/g of gelatin) was added to the solution under stirring for 40 minutes at 400 rpm. The whole process was performed at 50°C temperature to avoid the solidification of gelatin.



Fig 18. Schematic diagram of gelatin solution preparation.

2.2.3.3 Preparation of the PCL/GEL solution

PCL/GEL solution (4 ml) was obtained by dissolving the two polymers (15% w/v) with a PCL-GEL ratio of 80:20, in a 1:1 mixture of chloroform and formic acid, followed by 24 h stirring at 400 rpm. Then, GPTMS (3,68% v/v) was added to the solution under stirring for 40 minutes at 400 rpm.



Fig 19. Schematic diagram of PCL/GEL solution preparation.

2.2.3.4 Preparation of the PCL/GEL/nHA solution

The procedure for preparing the solution of PCL/GEL/nHA starts in the same way of the preparation of PCL/GEL blend.

PCL/GEL solution (4 ml) was obtained by dissolving the two polymers (15% w/v) with a PCL-GEL ratio of 80:20, in a 1:1 mixture of chloroform and formic acid, followed by 24 h stirring at 400 rpm. Then, GPTMS (3,68% v/v) was added to the solution under stirring for 40 minutes at 400 rpm.

While the solution was under stirring, nHA (20% w/w), was added and the blend was sonicated (Sonics Vibra-Cell sonicator) for 1 min.



Fig 20. Schematic diagram of PCL/GEL/nHA solution preparation.

2.2.4 Electrospun membranes

The four solutions obtained with the previous protocols were processed with the electrospinning technique with the instrument supplied by Electrospinning Linari Engineering (pictured in **Figure 21**).



Fig 21. Electrospinning instrumentation.

After placing the syringe containing the solution on the pump, one electrode was connected to the needle and the other two to the static collector (as shown in **Figure 22**), placed at a pre-determined distance.



Fig 22. Deposition of nanofibers during electrospinning process.

The parameters summarized in **Table 5** were set to obtain the electrospun membranes. For the solution of gelatin, the temperature of the electrospinning process was maintained at 50°C to avoid gelation. Nanofibers were deposited on a static collector coated with an aluminum sheet and on glass circular coverslips fixed upon the aluminum sheet with conductive double-sided adhesive tape.

	Voltage [kV]	Flow rate [ml/h]	Distance needle-collector [cm]
PCL	20	1,53	20
GEL	30	1,53	12
PCL/GEL	21	0,508	12
PCL/GEL/nHA	21	0,508	12

Tab 5. Parameters of the electrospinning process for the different solutions.

2.2.5 Membranes characterization

2.2.5.1 Field Emission Scanning Electron Microscope (FE-SEM)

The morphology of nanofibrous membranes was analyzed by FE-SEM.

The images of fibers, obtained with the different compositions, were captured at different magnifications (1000X, 2000X, 5000X, 10000X) with a voltage of 5 KV.

Four FE-SEM images for each different solution at the same magnification were analyzed trough the software ImageJ to measure the diameters (30 fibers each image).

2.2.5.2 Mechanical tests

MTS QTEST/10 is a mechanical testing machine used to evaluate tensile strength of materials with a static test. The machine, pictured in **Figure 23A**, applies an increasing load on the sample, that is placed between the grips (as shown in **Figure 23B**), the lower one unmovable while the upper one moves upward during the experiment, until failure.

The moving crosshead, with a 2 mm/min velocity, was fixed to a 10 N load cell that controls and records the tensile strength applied on the specimen while an extensometer measured the displacement. The samples were prepared cutting the membranes with a standard shape (two shoulders, which were gripped by the machine, and a smaller section in between) and size, measured by a digital caliper, 5 mm width, 13 mm initial length and a variable thickness (in the range of micrometers).



Fig 23. MTS QTEST/10 machine (A) and detail of a sample between the grips during the test (B).

Every second, the machine recorded the strength applied on, at least, 4 specimens for fibers type in function of the displacement by the software TestWorks 4. The acquired data were elaborated by the software Excel to obtain stress-strain curves and Young's moduli for each material.

2.2.6 In vitro cell culture tests

Both cell lines used in this work, PANC-1 and Mia PaCa, were thawed and cultured according to their specific culture protocols [58] [59] in RPMI medium supplemented with 10% FBS and Penicillin/Streptomycin.

2.2.6.1 Cell viability

For cell viability assays, cells (20000 cells/well) were cultured on the nanofibrous membranes deposited on glass coverslips, for 48 and 96 hours in 24 well plates. Plain coverslips were used as controls. All experiments were performed in triplicate for both cell lines. Cell-Title-Glo (CellTiter-Glo[®] Promega) assay, that is schematized in **Figure 24**, was used to determine the number of viable cells present on the membranes. This method determinates the number of viable cells based on estimate of ATP, indicator of metabolically active cells. At the pre-determined time points, samples were washed twice with PBS and the coverslips were transferred to a clean 24 well plate. Cell-Title-Glo

reagent, prepared according to manufacturer's protocol, was, then, added to each well (100 μ l/well) and the plate was stirred on an orbital shaker for 5 minutes in the dark, to induce cell lysis [60]. The sample volume is, then, transferred to a white opaque-walled 96 well plate, allowed to set for a few minutes to stabilize the luminescent signal and read with a Synergy HTX plate reader (pictured in **Figure 25**).



Fig 24. Schematic diagram of luminescent cell viability protocol.

Additional controls tested were background luminescence, obtained by testing a well containing only Cell-Titer-Glo, and membrane intrinsic signal, determined by testing PCL, PCL/GEL and PCL/GEL/nHA fibers collected on glass coverslips without cells.



Fig 25. Synergy™ HTX Multi-Mode Microplate Reader

Cell viability was expressed as a percentage of living cells detected on each substrate with respect to the number of living cells detected on the control coverslips, after subtraction of background contributions. All data are presented as mean ± standard deviation, calculated on at least 3 samples.

2.2.6.2 Cell morphology

Cell morphology on the substrates was evaluated at two different time points, 4 and 7 days, for both cell lines.

First, cells (20000 cells/well in 24 well plates) cultured on the nanofibrous membranes glass coverslips, were fixed with formalin free fixative for 20 minutes, followed by staining with Phalloidin (Flash Phalloidin[™] Green 488 supplied by BioLegend) and DAPI.

Phalloidin is a peptide used in fluorescence microscopy as a probe, due to its binding with F-actin, for imaging actin microfilaments in stabilized and permeabilized cells.

For Phalloidin staining, displayed in **Figure 26**, fixed cells were washed twice with PBS and permeabilized with 0.5% Triton X-100 solution for 10 minutes. Cells were, then, blocked with 5% fetal bovine serum (FBS) for 30 minutes and incubated with Phalloidin solution diluted (1:50) for 20 minutes under orbital shaking in the dark [61]. The sample was washed twice with PB, incubated with DAPI solution (1:10000 dilution) for 5 minutes in the dark and washed twice, again, with PBS. Samples were, then, mounted on microscope slides, using a FloroMont mounting medium (Sigma Aldrich).



Fig 26. Schematic diagram of cell staining for morphology.

Images were acquired on a Nikon Eclipse Ti2 microscope at magnifications of 10X, 20X and 60X. Images were processed through the software ImageJ to merge DAPI and FITC channels.

2.2.7 Nanoparticles preparation

To obtain a suspension of core-shell nanoparticles (NPs), which process is schematized in **Figure 27**, the polymer was first dissolved in acetonitrile (10 mg/ml).

1 ml of the polymer solution was, then, dropped in 2 ml of water containing 200 μ g of EGG-PG and 260 μ g of DSPE-PEG at 60°C, to avoid micelle formation. The suspension was further cooled by adding 1 ml of water and left under stirring at 400 rpm for 1 hour.

The NPs suspension was centrifuged at 3200 rpm for 12 minutes, using a 10 kDa-cutoff comlucular concentrator, washed twice with deionized water and collected for further analysis.



Fig 27. Schematic diagram of nanoparticles preparation.

2.2.7.1 Fluorescent nanoparticles

To obtain fluorescent nanoparticles, the same procedure (shown in **Figure 28**) was followed by adding 10 µg rhodamine-labelled lipids in the lipidic mixture.



Fig 28. Schematic diagram of fluorescent nanoparticles preparation.

2.2.8 Nanoparticles characterization

2.2.8.1 Field Emission Scanning Electron Microscope (FE-SEM)

The morphology of nanoparticles was analyzed by FE-SEM.

A small drop of the NPs suspensions was poured on polished silicon wafers and allowed to dry overnight. The sample was, then, mounted on aluminum stubs coated with platinum, and analyzed by FE-SEM at the magnifications of 50000X and 100000X with a voltage of 5 KV.

Two FE-SEM images were analyzed with the software ImageJ to measure the diameters of 30 nanoparticles.

2.2.8.2 Dynamic light scattering (DLS)

Dynamic light scattering is a non-invasive a technique for the characterization of particles, polymers or molecules in solution or in suspension. In this work, DLS was used to measure nanoparticles dimensions, size and polydispersity index, and the zeta potential.

The diameter measured is referred to as a hydrodynamic diameter because it represents the diffusion of nanoparticles within a fluid. It depends on the size of the particle core, on surface structure and on concentration and type of ions in the liquid part of the suspension.

The polydispersity index (PDI) has a value between 0 and 1 and it estimates the average uniformity of the suspension. The lower the value, the tighter the distribution and, on the contrary, the higher the value, the wider the distribution.

Zeta potential is a representation of the stability of suspension; its value rises with the speed of nanoparticles in presence of an electric field. A high zeta potential means a more stable suspension because repulsive forces overcome the attractive ones prevent the aggregation of suspension [62].

The sample was prepared pouring 10 µl NPs centrifuged suspension in 900 µl of water inside of specific cuvettes (Figure 29A and 29B) which are analyzed through Litesizer[™] 500, pictured in Figure 29. Size, PDI and zeta potential were tested with three different NPs suspensions.

The same analysis was repeated with fluorescent NPs centrifuged suspensions.



Fig 29. DLS, Litesizer[™] 500 with cuvette for size and PDI (20A) and zeta potential (20B) measure.

2.2.9 Cellular internalization of nanoparticles

Cells (100000 cells/well in 12 well plates) were cultured on the nanofibrous membranes glass coverslips for 7 days, followed by incubation with a suspension of fluorescent nanoparticles (0,5 mg/ml) for 24 hours. At the end of experimental timepoint, NPs internalization was evaluated by fluorescent detection, using a Synergy HTX plate reader (pictured in **Figure 25**). Briefly, cells were washed twice with PBS and detached by incubation with trypsin. Detached cells were collected by centrifugation (1500 rpm for 5 min), dispersed in sterile PBS, and analyzed by the plate reader using an excitation/emission filter of 530/590 nm. All experiments were performed in triplicate for both cell lines, using untreated cells and cells cultured in adherence conditions on the culture plate and treated with nanoparticles, as controls.

Chapter 3 – Results and discussion

3.1 – Characterization of synthesized nanoscale hydroxyapatite

Bioinspired nanoscale hydroxyapatite, obtained on the base of Gentile's protocol described previously, was evaluated by morphological and chemical characterizations.

3.1.1 Morphological characterization

Field emission scanning electron microscopy confirmed the nanometric dimension of the hydroxyapatite crystals, similar to the biological dimension of apatite [63]. As shown in **Figure 30A**, values of nHA diameters are in the range of a hundred nanometers.

Moreover, the reproducibility of the protocol was also confirmed and the results obtained from two separate syntheses evidenced no significance differences in term of neither morphology (**Figures 30B** and **30C**) nor average size: the medium diameter of the first synthesis was 116±38 nm while for the second one was 128±40 nm.



Fig 30. Diameters of hydroxyapatite nanoparticles (A) and FE-SEM images of synthesis_1 (B) and synthesis_2

3.1.2 Chemical characterization

3.1.2.1 Energy-dispersive X-ray spectroscopy (EDX)



Fig 31. EDX Spectrum of nHA crystal (on the left).

The EDX spectrum (**Figure 31**), shows the presence of three main peaks attributed to calcium (Ca), phosphorus (P) and oxygen (O), which are the constituent elements of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$.

3.1.2.2. X-Ray Diffraction Analysis (XRD)

Nanohydroxyapatite was tested before and after the sintering process.

There is a clear correspondence between the pattern of the samples reported in **Figure 32A2** and **32B2** and that of hydroxyapatite reported in **Figure 32A3** and **32B3***.



Fig 32. XRD spectrum of non-sintered nHA (A1) with related pattern (A2) compared with those of $Ca_{10}(PO_4)_6(OH)_2$ (A3)* and XRD spectrum of sintered nHA (B1) with related pattern (B2) compared with those of $Ca_{10}(PO_4)_6(OH)_2$ (B3)*.

*Data supplied by Prof. Marta Miola and Prof. Enrica Vernè – DISAT, Politecnico di Torino

Comparing XRD spectrums of both samples (**Figure 33**), an increase in the crystallite size of sintered nHA becomes evident, in accordance with the experimental work of Gentile and collaborators [53].



Fig 33. Diffractograms of non-sintered nHA (on the top) and sintered nHA (on the bottom)

Diffractogram of sintered nHA has a much cleaner spectrum with sharper diffraction peaks and a reduction of peaks width, indicators of presence of crystalline phases in the sample.

3.2 – Characterization of membranes

3.2.1 Morphological characterization

The morphology of fibers was evaluated by images obtained with field emission scanning electron microscopy; **Figure 34** shows that fibers with random aligned were obtained with all tested materials. Fibers appear quite uniform in size with little defects.



Fig 34. FE-SEM images of fibers realized with different solutions at increasing enlargements.

The values of diameters, shown in **Figure 65**, were in the range of nanometers except for the fibers realized with PCL/GEL blend reinforced with nanohydroxyapatite, which have an average diameter around $1 \mu m$.



Fig 35. Medium diameter of PCL, gelatin, PCL/gelatin and PCL/gelatin/nHA fibers.

According to literature, membranes constituted by PCL/GEL/nHA fibers had a larger diameter, with a medium value of 1039±435 nm, due to the doping with hydroxyapatite nanoparticles which tend to agglomerate inside the fibers [64].

PCL fibers had the second larger diameter, 659±174 nm, because of the viscosity of PCL solution, while gelatin fibers had the smallest one (265±93 nm) and fibers obtained with the PCL/GEL blend a value of 505±188 nm was in the middle. This can be explained by the properties of the utilized polymers: PCL is a synthetic polymer that does not produce ions when dissolved in organic solvent like chloroform; gelatin is a polyelectrolyte polymer that releases carboxyl and amino groups in acid or neutral pH solutions. Therefore, gelatin solution has a larger number of charged groups which increase the conductivity of the solution, leading to fibers with smaller diameter after electrospinning. The addition of gelatin in the blend caused a reduction of PCL/GEL fibers dimension compared to ones obtained with bare PCL [65].

3.2.2 Mechanical characterization

Stress strain curves (**Figure 36**) obtained by tensile strength testes with MTS QTEST/10 showed the greatest resistance for PCL/GEL/nHA fibers due to the contribution of hydroxyapatite in strengthening the structure. Gelatin specimens had a significantly lower mechanical resistance, due to the poor mechanical properties of this polymer, resulting in the failure of samples after around 13% of the obtained strain. PCL and PCL/GEL substrate exhibited an intermedium behavior: they tolerated higher mechanical stress than gelatin, achieving greater strain % than gelatin fibers.



Fig 36. Stress-Strain curves for PCL, gelatin, PCL/gelatin and PCL/gelatin/nHA fibers.

PCL, being an elastic polymer, presented the lowest Young's modulus, 13±4 MPa, and the PCL/GEL blend had a similar value (14±4 MPa), as shown in **Figure 37**. Gelatin showed the highest elastic modulus of 46±10 MPa, confirming the values reported in literature [26]. The Young's modulus of PCL/GEL/nHA substrate (31±11 MPa) is situated in the middle because of the increase in rigidity caused by hydroxyapatite.



Young's Modulus

Fig 37. Elastic modulus of PCL, gelatin, PCL/gelatin and PCL/gelatin/nHA fibers.

Among the material tested, PCL/GEL/nHA blend showed the greatest resistance, as the bone is the hardest tissue of the body. The values of elastic modulus obtained with different fibers compositions are placed in the middle between soft tissues, such as liver and lung, values and that of hard tissue like the bones, as shown in **Table 1**.

3.3 - In vitro cell tests

3.3.1 PANC-1

Cell viability tests evidenced a good viability on all substrates for PANC-1 cell line at the 48 h timepoint, which showed similar values to the control sample (glass coverslip without fibers). No differences were detected among samples and between samples and controls. At the 96h timepoint (**Figure 38**), only gelatin nanofibers offered similar values as the control sample. A relevant reduction in the percentage of surviving cells compared to the control was noticed on all other substrates at the 96h timepoint.



Fig 38. PANC-1 viability at 48h and 96h timepoints.

Images acquired by fluorescent microscope (**Figure 39**) showed that PANC-1 cells tend to grow in clumps, the size of which increased over time. This may explain the similar trend in cell viability at the early timepoint, where cells displayed a similar viability regardless of the substrates. Cell clumps begin to spread after the 4 days timepoint. During this time frame the effect of substrate on cell viability becomes more evident, as the substrate composition/mechanical properties may affect cell spreading.



Fig 39. Merging of DAPI and FITC channels of images captured at 20X.

Cells grew on all substrates tested with a higher propensity for gelatin fibers probably due to the good biocompatibility of this material and for PCL/GEL/nHa membranes because of the greater stiffness introduced by hydroxyapatite.

The interaction between cells and substrates was good with the presence of several definite clumps. Representative images of PANC-1 cell clumps on selected substrates are shown in **Figure 40** and at higher magnifications of 60X in **Figure 41**.



Fig 40. PANC-1 cultivated on PCL/GEL/nHA substrate at 7 days timepoint, captured at 20X.



Fig 41. PANC-1 cultivated on PCL/GEL substrate at 4 days timepoint, captured at 60X.

3.3.2 MIA PaCa

MIA PaCa cultivated on gelatin fibers turned out to have a 50% higher viability than cells grew on the same substrate at 48 h timepoint (**Figure 42**).

At both timepoints the highest percentage of cell surviving is recorded on gelatin substrate. PCL/GEL blend and PCL/GEL doped with hydroxyapatite showed good viability values, specially at the 96h timepoint.



Fig 42. MIA PaCa viability at 48h and 96h timepoints.

Images acquired by fluorescent microscope (Figure 43) displayed MIA PaCa cells spread on several levels growing inside the fibers.



Fig 43. Merging of DAPI and FITC channels of images captured at 20X.

Cellular density was high on all substrates, but particularly, again, on gelatin and PCL/GEL/nHa fibers. Random PCL/GEL fibers appear clear in **Figure 44**, while in **Figure 45** the absence of fibers depend on complete degradation of gelatin after 7 days.



Fig 44. MIA PaCa cultivated on PCL/GEL substrate at 4 days timepoint, captured at 60X.



Fig 45. MIA PaCa cultivated on gelatin substrate at 7 days timepoint, captured at 20X.

At the 48h timepoint, PCL fibers showed a more evident difference in survival between the two cell lines: MIA PaCa displayed a viability around 50% lower than PANC-1. The gap was less marked for other three types of membranes.

Gelatin fibers offered the most suitable substrate for both cell lines, due to the good biocompatibility of gelatin. In fact, the percentage of cell viability was >91% for MIA-PaCa and even higher for PANC-1. This behavior matched with that reported in literature [44].

The substrate realized with PCL/GEL/nHA fibers also shows a good behavior in terms of cell viability, probably because of the presence of gelatin that made the blend more hydrophilic and of the support of nanohydroxyapatite, which may facilitate cell attachment or spreading by strengthening the support [42]. The PCL/GEL blend had a slightly lower cell viability, perhaps attributable to the lack of the nanohydroxyapatite component.

PCL fibers were the substrate less suitable for cell survival because of the hydrophobic nature of PCL polymer, as also reported in literature [66].

After 96 hours of cell culture a reverse trend occurred with a higher percentage of cell viability on the fibers for MIA-PaCa than for PANC-1. The gelatin fibers were confirmed as the ideal substrate to grow cells.

Otherwise, the behavior of each substrate reflected that found at 48h: both PCL/GEL/nHA and blend PCL/GEL had intermediate performances and PCL reasserted its poor behavior as culture support.

On a morphological point of view, PANC-1 tend to grow in clumps, as evidenced by the presence of separated areas with high cellular density, while the growth of MIA PaCa over the substrates was more uniform [50] [51].

3.4 – Characterization of nanoparticles

3.4.1 Morphological characterization

Field emission scanning electron microscopy (Figure 46) confirmed the nanometric dimensions of NPs and their spherical shape.



Fig 46. FE-SEM image of nanoparticles.

The size of hybrid polymer/lipid nanoparticles were in the range of nanometers, with a medium diameter value of 297±101 nm.

Hydrodynamic diameter confirmed the nanoscale dimensions of NPs and was reflected by values of literature [67]. The difference between the medium diameters of NPs (158±9 nm) and rhodamine labelled NPs (144±15 nm) was minimal (**Figure 47**), suggesting that the surface modification with the model fluorophore does not affect the properties of the particles.

Similarly, the difference between polydispersity index (PDI) values of 0,08±0,03 for NPs and 0,13±0,01 for fluorescent NPs was negligible (**Figure 47**). The low value of PDI indicates that nanoparticles had comparable dimensions and unimodal distribution (**Figure 48**)



Fig 48. Distribution of three repetitions of diameter measures for NPs (A) and rhodamine NPs_R (B).



Fig 47. Size and PDI of NPs and rhodamine NPs_R.

Zeta potential provided negative values for both types of nanoparticles but with a significative difference in the magnitude (**Figure 49**). Zeta potential for NPs varied between -43 and -64 mV stating a good stability for the solution, while the values for rhodamine NPs ranged between -9 and -41 mV, so fluorescent nanoparticles had more variable results, coherently with the presence of rhodamine on the surface [62].



Fig 49. Zeta potential of NPs and rhodamine NPs_R.

3.5 – Internalization of nanoparticles

Cells cultured on gelatin fibers showed a higher internalization of nanoparticles (**Figure 50**), probably due to the higher cell viability on this substrate. A good cellular internalization of NPs is recorded on PCL/GEL and PCL/GEL/nHA substrates.

The percentage of cellular internalization of NPs, for cells grown on nanofibrous substrates compared to the control, same cell line cultivated in adherence conditions on the culture plate treated with nanoparticles, is higher for PANC-1 than MIA PaCa.



Fig 50. Cellular internalization of NPs

Chapter 4 – Conclusions and future developments

The realization of membranes with the capability to mimic the extracellular matrix of human organs is one of the aims of tissue engineering. The complexity and diversity of tissues in their structure and mechanical characteristics require the use of blend constituted by synthetic and natural polymers. The combination of features belonging to both categories permits to merge the advantages and to minimize the disadvantages of both, by realizing substrates with appropriated mechanical properties and stability and, also, with a good interaction with cells.

Here, the choice of adding a mineral component to the polymers blend permitted to modulate the mechanical properties and to obtain a stiffer substrate, although a more complete mechanical characterization of fibers is still needed. Moreover, the electrospinning parameters set to obtain the nanofibers could be modified to obtain substrates of varying thickness, as this parameter may also affects the behavior of cells.

Both pancreatic cell lines, PANC-1 and MIA PaCa, showed a good viability at the two timepoints tested (48h and 96h), with a particularly good behavior on the PCL/GEL/nHA substrate and on the gelatin nanofibers. To further confirm these results, other cancer cell lines, obtained from different primary tumors could be cultured on these substrates. Moreover, the viability could be monitored overtime for longer timepoints to provide additional information on the long-term behavior. Lastly, the comparison with normal cells, native of tissue which ECM we are going to mimic, could determinate the real impact of the mechanical properties of the substrates on the tumor cells, compared with the normal cells.

Nanoparticles internalization suggests that this parameter may be affected by properties of substrate. Further studies should focus on the response of the cells to treatment, by testing drug loaded NPs with the aim to assess whether the response to treatment may be linked to properties of the substrate on which cells are grown.

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