Fabrication of Nanostructured Poly-ε-caprolactone 3D Scaffolds for 3D Cell Culture Technology

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ABSTRACT

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Tissue engineering is receiving tremendous attention due to the necessity to overcome the limitations related to injured or diseased tissues or organs. It is the perfect combination of cells and biomimetic-engineered materials. With the appropriate biochemical factors, it is possible to develop new effective bio-devices that are capable to improve or replace biological functions. Latest developments in microfabrication methods, employing mostly synthetic biomaterials, allow the production of three-dimensional (3D) scaffolds that are able to direct cell-to-cell interactions and specific cellular functions in order to drive tissue regeneration or cell transplantation.

The presented work offers a rapid and efficient method of 3D scaffolds fabrication by using optical lithography and micro-molding techniques. Bioreorable polymer poly-ε-caprolactone (PCL) was the material used thanks to its high biocompatibility and ability to naturally degrade in tissues. 3D PCL substrates show a particular combination in the designed length scale: cylindrical shaped pillars with 10µm
diameter, 10µm height, arranged in a hexagonal lattice with spacing of 20µm were obtained. The sidewalls of the pillars were nanostructured by attributing a 3D architecture to the scaffold. The suitability of these devices as cell culture technology supports was evaluated by plating NIH/3T3 mouse embryonic fibroblasts and human Neural Stem Cells (hNSC) on them. Scanning Electron Microscopy (SEM) analysis was carried out in order to examine the micro- and nano-patterns on the surface of the supports. In addition, after seeding of cells, SEM and immunofluorescence characterization of the fabricated systems were performed to check adhesion, growth and proliferation. It was observed that cells grow and develop healthy on the bio-polymeric devices by giving rise to well-interconnected networks. 3D PCL nano-patterned pillared scaffold therefore may have considerable potential as effective tool for applications in tissue engineering.
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I would like to express my gratitude to my supervisor, Prof. Enzo Di Fabrizio, for giving me the possibility to live this beautiful experience at KAUST and discover my passion for the world of research.

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LIST OF ABBREVIATIONS

(3D) - Three-dimensional
(PCL) - Poly-ε-caprolactone
(SEM) - Scanning Electron Microscopy
(hNSC) - Human Neural Stem Cells
(ECM) - Extracellular matrix
(Si) - Silicon
(2D) - Two-dimensional
(PHEMA) - Polyhydroxyethylmethacrylate
(PNIPAAm) - Poly(N-isopropylacrylamide)
(PVA) - Polyvinyl alcohol
(PLA) - Polylactide
(PGA) - Polyglycolide
(PLGA) - Poly(lactide-co-glycolide)
(PDO) - Polyphosphazene, polydioxanone
(ROP) - Ring-opening polymerization
(UV) - Ultraviolet
(CAD) - Computer-assisted design
(MEMS) - Micro-electromechanical systems
(EBL) - Electron Beam Lithography
(DRIE) - Deep Reactive Ion Etching
(PDL) - Poly-D-lysine
(ATCC) - American Type Culture Collection
(DMEM) - Dulbecco's Modified Eagle’s Medium
(PFA) - Paraformaldehyde
(PBS) - Phosphate Buffered Saline
(BSA) - Bovine Serum Albumin
(GFAP) - Glial Fibrillary Acid Protein
(DIV) - Days in vitro
Chapter 1

Introduction

Nowadays many diseases and accidents cause several damages to organs and tissues, representing a big threaten for the life. Autografting and allografting are two of the main techniques used at the time for tissue repair [1]. Both of them, however, show some limitation in their applications. An autograft is the fastest and safest-healing tissue that can be used, but it requires the creation of a second surgical site from which the patient has to recover. An allograft is a tissue that comes from a donor; it could take long time to incorporate into the recipient’s body, it could lead to either an immune response or danger of disease transfer and there could be issues in the availability of tissues.

Recently tissue engineering became a promising field with the aim of fabricating biological alternatives in order to develop new methods and devices for repairing or replacing tissues and damaged cells. It can be defined as the “application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue
function” [2]. The success of this discipline is mostly based on the combination of two important elements: cells and scaffolds. There are three main approaches in tissue engineering. Genetically manipulated cells can be used to substitute some defective cells in order to restore the original function. Tissues carrying particular substances (growth factors, adhesive proteins, etc.) can be delivered to specific locations. Cells seeded *in vitro* or coming from host tissues *in vivo* can grow in three-dimensional devices. Lately stem cells have shown unique capabilities in multilineage differentiation and self-renewal becoming a new important cell source; on the other hand nano-materials and polymeric ones emerged as promising candidates for the production of scaffolds able to well reproduce the natural extracellular matrix (ECM). It has been observed how the organization and interaction between cells and substrate can greatly affect many cellular processes such as adhesion, growth, proliferation, migration and death. Therefore, the success or failure of medical tools depends mainly on the good interactions between cells and material that is going to be implanted. Since cell response is influenced by several chemical and physical properties of the biomaterial surface like chemical composition, surface energy and charges, it has been established that topography is one of the most important cues for cells. It has become clear that micro-topography [3, 4] and, more recently, nano-topography [5, 6] are able to guide cells behavior properly. Consequently, the behavior can be tailored by modifying nano- and micro-scale
characteristics related to topology and geometry [7]. It is important to design the device so that it is closer to the molecular/cellular scale by establishing specific geometries to the chosen surface that determines the most suitable physicochemical parameters of the substrate itself. In some recent studies, micro- and nano-topographical patterns working as contact guidance for cells are a promising perspective especially for those cases where functional tissue can be achieved thanks to the guidance of cell orientation [8, 9]. Furthermore it has been found that wettability and roughness are two of the most important factors that influence the cell biological reactions at the surfaces [10]. It has been shown how cultures of astrocytes [11] and neurons [12, 13] can grow on micro-grooved platforms characterized by different depths. Speaking of superhydrophobicity, it has been recently developed a validated method that allows neurons and astrocytes to adhere to the sidewall of nanostructured silicon (Si) pillars by forming suspended and mature networks [14].

Biomaterials and latest improvements in microfabrication technologies play a crucial role in the production of nano- and micro-structured devices, by reproducing properly the natural topography leading to new tissue formation. Many approaches have been developed for the creation of 3D scaffolds made of biomaterials; between the huge variety of those materials employed for biomedical and regenerative medicine applications, both natural and synthetic polymers have been widely investigated.
For the production of nano- and micro-topography on biomaterial surfaces, several methods have been employed as tools for the control of tissue regeneration. Such techniques may lead to disordered topography characterized by random organization and orientation or to ordered topography with regular organized patterns. Some of the most effective techniques that allow to obtain patterns with random organization are colloidal lithography, chemical etching, polymer demixing. On the contrary, methods mostly used to produce regular geometries employ different sources of radiation such as electrons, ions and protons; some examples are projection lithography and scanning beam lithography [15]. Photolithography is the most common type of projection lithography, where the diffraction of light is used to expose a photo-activated film of resist. If combined with appropriate methods of fabrication, it allows the creation of devices characterized by superficial features with high resolution and size ranging from several nanometers (less than 50 nm) to few hundreds of microns [16].

The main goal of this project is to describe a validate fabrication method for the production of three-dimensional Poly-ε-caprolactone (PCL) scaffolds for cell seeding technology and their characterization. They are characterized by a nano-structured pattern that is able to well recreate the ECM function in a spatially organized system. The techniques used for the development of this
work are photolithography and micro-molding methods; this unique fabrication approach allows the realization of scaffolds that are able to provide an efficient surface area for guidance of cells growth and adhesion. Thanks to the resorbable nature of the PCL, minimum mechanical stability is provided in order to support a good cellular growth by making the scaffold a promising tool for future studies and implantations.
Chapter 2

Background

2.1. Importance of tissue engineering

The lost and damage of tissues or organs, caused by many traumas or simply by the aging, is one of the main concerns affecting the medical field since it is an expensive and difficult problem to solve. This led to the development of tissue engineering that aims to create biological substitutes for repairing or replacing diseased tissues and organs [17].

The word “tissue” refers usually to a bunch of the same type of cells with their extracellular matrix that plays an important role in tissues as it works as both supporter and regulator of cells behavior. The term “engineering” means working artfully to bring something about and to guide the course of. The fundamental concept behind tissue engineering is to produce devices basically by following two ideas: implanting an a-cellular scaffold in vivo that is able to guide the patient’s cells to repair the damaged tissue; creating a scaffold that is able to guide the autologous cells in vitro to expand. These two approaches avoid some drawbacks such as lack of donor tissue, no presence of foreign body at the targeted site since the scaffold should
degrade at certain point, consequently there’s no chronic inflammation, no rejection and transfection problems thanks to the use of autologous cells. In Figure 2.1 a schematic diagram of the different steps from the scaffold fabrication till the *in vivo* implantation is shown.

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**Figure 2.1.** Schematic representation of the main processes that describe what is tissue engineering from the fabrication of the scaffold, cells isolation, *in vitro* seeding of cells to *in vivo* implantation of the device.
By considering the cellular aspect, it is important to choose very carefully the source and type of cells before culture or implantation. The choice must be done in order to guarantee sufficient supply and to avoid eventually the presence of pathogens and contamination. Once the cells are collected, they can be cultured in vitro for certain time in which they are kept alive and can expand. After that cells must be seeded onto the scaffold. It is essential for the scaffold to provide the mechanical and chemical cues the cells require to preserve their function. In other words, the success of the scaffolds in turning into integrated tissue depends on the entity of cell-adhesion sites distributed on the device surface with appropriate density; this will promote cell migration [17]. Once cells have populated the scaffold they start to receive the relevant cues and stimuli that make cells to proliferate and differentiate thanks to growth factors, cytokine and integrins with which cells bind to the ECM [18, 19]. That’s basically how the neotissue is produced and can be modeled in function of the mechanical environment.

The other major aspect in tissue engineering is the scaffold itself, its design, molecular treatment or surface features it may carry and last but not least the biomaterial it is made of that must be biocompatible and biodegradable.

To summarize, the most promising approach in tissue engineering is to let cells grow on 3D biodegradable scaffolds, which operate as temporary supports that promote cell adhesion, proliferation and differentiation.
After that, those scaffolds should degrade by leaving the new healthy regenerated tissue in the targeted location. The big challenge for biomedical devices is in the design and fabrication of biodegradable and biocompatible tools with such properties that would be able to support cell growth and all cellular mechanisms and facilitate the formation of the ECM [17, 20]. Fundamental criteria for the development of those devices involve the selection of materials with specific internal architecture, superficial features and mechanical properties compatible with those of the host tissue such as elastic modulus, strength, sterilizability and biocompatibility with expected degradation rate [21, 22]. The ECM is able to influence aspects of the cells behavior, since in a native tissue environment it is in contact with cells and works either as structural framework and cells activity regulator [23]. Therefore, when designing a device, it is essential to closely reproduce the morphology, chemical composition and functional groups of the natural environment, by taking into account the desirable properties the scaffold should have depending on the specific application. It is important it can mimic functionally and architecturally the ECM as it is well known that topography of the material affects cellular activity [24]. Moreover, the substrate needs to be porous, biocompatible and bio-resorbable with high surface area so that cells can easily attach and penetrate; cell-to-cell and cell-to-matrix communication and exchange of nutrients and degradation products as well are allowed.
2.2. Two-dimensional vs. three-dimensional devices

Living organisms are characterized by a cell’s environment, a 3D architecture where cells interact not only with each other, but also with the ECM. Nonetheless cell culture studies have mostly been performed on two-dimensional (2D) substrates such as glass or plastic plates/flasks because it was found high cell viability and convenience in addition to great understanding of basic cell biology. On the other hand, 2D surfaces are not able to reproduce the complex 3D in vivo environment since cells growing on them are forced to adhere to those flat and rigid substrates by causing compromised functionality and altered metabolism [25, 26]. 3D scaffolds have been recently introduced in order to overcome the limits deriving from 2D cell culture systems so that the gap between cell cultures and physiological tissues can be downed. Comparisons among 3D systems and 2D monolayer cultures showed that, the physiology of different cell types varies considerably in the first environment. Particularly, physicochemical modifications could make the scaffold itself able to offer several features to match tissue specific applications [27, 28]. Despite 3D substrates have shown to fit better the in vivo architecture, either advantages and disadvantages can be highlighted for both 3D and 2D devices. In Table 2.2 main advantages and disadvantages in both cases are summarized.
Table 2.2. Summary of the main characteristics related to 2D and 3D cell cultures.

<table>
<thead>
<tr>
<th>2D vs. 3D CELL CULTURES</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D CULTURES</td>
<td>• Environmental control</td>
<td>• Deficiency in cell-to-cell/cell-to-matrix interactions</td>
</tr>
<tr>
<td></td>
<td>• Easier cell observation, manipulation and measurement</td>
<td>• Inherent representation of <em>in vivo</em> systems</td>
</tr>
<tr>
<td></td>
<td>• Existence of huge body of literature to compare results with</td>
<td>• Less compatibility with <em>in vivo</em> systems</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Majority of cells surface exposed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Altered drug sensitivity</td>
</tr>
<tr>
<td>3D CULTURES</td>
<td>• Cells in proximity with one another on all sides</td>
<td>• Diffusional transport limitations: essential nutrients may not reach each cell; toxic waste products may accumulate within scaffold space</td>
</tr>
<tr>
<td></td>
<td>• Cells able to extend in all directions</td>
<td>• Culture dependent alterations in gene expression, cell proliferation, viability, productivity and product quality due to nutrient deprivation</td>
</tr>
<tr>
<td></td>
<td>• More accurate representation of <em>in vivo</em> cytoarchitecture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• More <em>in vivo</em>-like cell behavior</td>
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### 2.2.1. Advantages of 3D scaffolds

The importance of working with cellular systems that are able to mimic the ECM determined the transition from cell monolayers to 3D cultures. The ability of single layer-based
cell cultures to predict cellular responses of living organisms is limited since they represent very simplified conditions of the cellular environment. Indeed, 2D substrates miss cell-to-cell and cell-to-matrix communication, mechanical and chemical cues, specific architecture of tissues. On the contrary, many studies showed that growing cells on 3D scaffolds reduce the big gap between cell cultures and physiological tissues. Three-dimensional strategy has the great potential to improve not only the cell-based literature but also the development of new cell lines and consequently devices or implants, quantitative analysis methods of biological systems that involve even 3D imaging techniques, cell-based drug and toxicity screenings that could decrease the number of animals using for test purposes [29]. As already said, cells interact with the environment and other cells thanks to biochemical and mechanical cues. 3D cell cultures have the potential to recreate such physiological communication by reproducing the specificity of real tissues better than 2D cultures. The clear understanding of the design parameters for survival, growth and functionality of cells determines the successful applications of tissue engineering tools. The development of 3D cellular architectures in vitro allows a good understanding of the appropriate parameters that regulate the cell-to-cell support, chemical support, matrix mechanical factors and mass transport limitations. It’s for this reason 3D cultures are used in many studies, from cell biology to cell adhesion, cell migration and epithelial
morphogenesis. Moreover three-dimensional technology could also be integrated with non-biological engineered components or electrical, chemical and microfluidic control by forming bio-hybridized systems. It has been demonstrated how the role of the ECM largely affects in tumorigenesis: epigenetic processes are triggered in the cell when the normal healthy microenvironment is perturbed; it could cause neoplastic transformations [30]. It has found that fibroblasts migrating on 2D flat substrates have different shape and distribution of adhesion proteins compared to fibroblasts on 3D devices where migration strategies are set depending on specific biological situations [31, 32]. Other experiments demonstrated that the ECM influences the structure of the chromatin in mammary epithelial cell lines and, therefore, the gene expression: cells cultured in 3D scaffolds have several gene expression levels compared with the 2D ones [33]. A 3D scaffold works either to provide a high surface area for growth and migration, which can be tailored to support cell behavior and to protect the cells from environmental perturbations. On a 3D environment cell-to-cell/cell-to-matrix interactions can be experienced in all spatial directions in different types, quantity and distributions, while they are constrained on planar configurations: those interactions can be experienced only on a single plane. This phenomenon could have some implications on morphology, growth, proliferation, viability, gene and protein expression and in response to mechanical or biochemical stimuli [31,
33, 34, 35]. Specifically, cells show different morphology and cytostructure by growing in 3D versus 2D environment. It was observed that cells on scaffolds have a more spherical shape than 2D cultures, and the outgrowth can occur in all directions in case of neurons and other processes. On the other hand, cells on 2D devices present an unrealistic and flattened shape [36]. By summarizing, cells growing in planar structures have different morphology [26], proliferation rates [36], migration [37], differentiation [19], gene expression [33], cellular signaling [38] and pathological susceptibility [39]. For instance, it has been demonstrated that 3D neuronal cell culture system result in longer neuritis outgrowth, greater cell survival and network formation in comparison with 2D cultures [40]. In a comparison between embryonic mesencephalon tissue in 2D and 3D architecture, it was found that cell death occurred mostly in monolayer cultures while 3D cultures in collagen gels survived much more [41]. In addition neural culture 3D systems are more suitable for electrophysiological studies than the flat rigid counterparts [42].

2.2.2. Disadvantages of 3D scaffolds

3D in vivo devices have some limitation and design consideration that compromise their function. Those restrictions in 3D cultures may involve diffusional transport problems that cause the accumulation of toxic waste
products next to the cells and many difficulties to the essential nutrients in reaching all cells. It follows a significant deviation from *in vivo*-like behavior resulting in altered gene expression and harmful effect on proliferation, viability, synthesis and function of proteins [43]. Nonetheless, by designing the scaffold with the appropriate degree of porosity, sufficient mass transport and proper cell and matrix elements can be regulated. Thus, 3D models may be able to reproduce more accurately the natural tissues than 2D ones.

2.3. Biomaterials and biocompatibility and biodegradability

Nowadays third-generation biomaterials play an important role in many techniques of the tissue engineering. For example, they can work as substrate for the adhesion and migration of cell cultures. Therefore, the resulted scaffold can be combined with specific cell types as either vehicle for cells or drug delivery system in order to activate specific localized functions [44]. At the beginning the main reason for using biomaterials was because of their great compatibility with biological systems by minimizing the body’s immune response to foreign materials. This purpose is still pursued today since the biological inertness of biomaterials is sometimes synonymous of non-recognition.
by the body. This can cause chronic inflammation or fibrous tissue encapsulation, which consequently affect the long-term biocompatibility and mechanical performances of an implant. Then second-generation biomaterials were studied: the goal was to enhance biomaterial-body interface by improving biological recognition. Bio-resorbable materials were exploited and bioactive components were used in an attempt to control biomaterial behavior in a physiological environment. The development of third-generation biomaterials is now focused on the design of biomimetic materials able to produce specific cellular response and guide the formation of new tissue through molecular recognition mechanisms. This particular approach, based on a combination between scaffolds with molecules of the ECM, allows a better control of the proliferation, migration, differentiation and survival of cells [45].

Biomaterial is an essential tool for tissue engineering though. Its task is to facilitate the regeneration or replacement of tissues, organs or functions; it should be designed for desired applications by being in contact with physiological tissues without producing any significant adverse response to its host. Designing the final structure of the biomaterial is fundamental, cause it determines the reaction of the host to the extraneous material that is responsible for the success or the failure of the device. Different variables that can influence the host response to the material are listed in Table 2.3. Particularly the requirements to be considered when choosing a biomaterial
are biocompatibility, biodegradability, appropriate physical and mechanical properties, processability and sterilizability.

Table 2.3. List of variables that can affect the material and host interactions.

<table>
<thead>
<tr>
<th>VARIABLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Morphology, micro-/nano-structure, bulk material composition</td>
</tr>
<tr>
<td>• Water content, hydrophobic/hydrophilic balance</td>
</tr>
<tr>
<td>• Macro-/micro-/nano-porosity</td>
</tr>
<tr>
<td>• Surface topography</td>
</tr>
<tr>
<td>• Surface chemical composition, surface molecular mobility, chemical gradients</td>
</tr>
<tr>
<td>• Surface electrical/electronic properties</td>
</tr>
<tr>
<td>• Surface energy</td>
</tr>
<tr>
<td>• Crystallinity and crystallography</td>
</tr>
<tr>
<td>• Elastic constants</td>
</tr>
<tr>
<td>• Debris release profile</td>
</tr>
<tr>
<td>• Degradation profile, degradation product and toxicity (for polymers)</td>
</tr>
<tr>
<td>• Additives, catalysts, contaminants and their toxicity (for polymers)</td>
</tr>
<tr>
<td>• Corrosion parameters, ion release profile, metal ion toxicity (for metals)</td>
</tr>
<tr>
<td>• Dissolution/degradation profile, degradation product toxicity (for ceramics)</td>
</tr>
</tbody>
</table>
“Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy” [46]. Moreover, in biodegradable devices mechanical properties and degradation rate are to be compatible with the need; it means that the material should show enough resistance in order to accomplish its function.

2.3.1. Polymer-based scaffolds materials

Among the big variety of biomaterials, polymers that present bio-stable, bio-resorbable or partially biodegradable nature are mostly chosen. Polymers are organic materials produced throughout polymerization from single molecules called monomers. Biopolymers can be fabricated easily in different states (solids, films, hydrogels, viscoelastic materials). They have density similar to the natural tissues and their physical, chemical and mechanical properties can be tailored by creating new blends with other monomers. In particular they can get different structures like alternating, random, branched or block copolymers; they can build interpenetrated networks; they can form compatible or non-compatible mixtures. The main disadvantages related to the
use of polymers are: low elastic modulus that represents a limit in the applications where a big load resistance is required, especially for degradable polymers; the polymerization itself sometimes makes them biodegradable; difficulty in obtaining biopolymers without using additives, antioxidants, plasticizers. For biomedical applications, polymers can be classified as biodegradable or non-degradable depending on their behavior after implantation or when they are in contact with biological fluids. Once implanted, biodegradable polymers undergo chemical/physical transformation resulting in the disappearance with time. Great attention is provided to bio-resorbable polymers since they are subjected to progressive degradation within biological systems by avoiding toxic and rejection reactions. It is important to identify the main factors that affect the degradation rate and its mechanisms: they must be set according to the specific clinic application. In the human body, the principal degradative agents that are able to modify polymeric materials are water, enzymes, oxygen and its reaction products found in the organism. The most important degradation mechanism is the hydrolysis; its rate depends on the nature of the polymer (on the kind of bonds into the polymeric chain), on the presence of heteroatoms in the polymeric chain and on the water concentration available for the hydrolytic process. The water diffusion coefficient (between $10^{-6}$ and $10^{-9}$ cm$^2$/s) and the water absorption become important. In particular polymers that absorb less than 1 wt.% of water are said hydrophobic,
between 1 and 10 wt.% of water are said partially hydrophilic, greater than 10 wt.% of water are said hydrophilic. The process of water diffusion in polymers is very complex and depends on both kinetic and thermodynamic factors. It is well known that the presence of anions and cations in the water diffuses in the polymeric material, significantly changes the hydrolysis rate: Na⁺, K⁺, Ca²⁺, Mg²⁺, Fe²⁺, Fe³⁺, Cl⁻, HCO₃⁻, H₂PO₄⁻, PO₄³⁻, HSO₄⁻ ions can increase it by 3-5 times. Other important factors that influence the kinetic of the hydrolysis are the crystallinity of the polymer: amorphous zones are involved more rapidly into the process because they are more accessible to the water molecules; the temperature: when it overcomes the glass transition temperature of the material there’s greater chain mobility which encourages the water penetration; the chemical structure: hydrophilic and hydrophobic groups can make the process easier or harder; the morphology of the surface: inhomogeneities, rugosities and cavities accelerate the degradation process. The parameters that affect the hydrolytic degradation can be macro or microstructural and are summarized in Table 2.3.1-1.

The resorbable polymers can also undergo to enzymatic degradation that is always connected to the hydrolysis and depends on the composition of the polymer itself. Enzymatic degradation can be stimulated by introducing, in the polymeric chain, specific sequences of amino acids that can be recognized by certain enzymes.
Even some radicals in the human body can start degradation processes of the polymers inserted.

Table 2.3.1-1. Main factors affecting the degradation rate of polymers.

<table>
<thead>
<tr>
<th>MICROSTRUCTURAL FACTORS</th>
<th>MACROSTRUCTURAL FACTORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Chemical structure</td>
<td>• Dimension and geometry of the implant</td>
</tr>
<tr>
<td>• Chemical composition of the polymer</td>
<td>• Weight/surface ratio</td>
</tr>
<tr>
<td>• Distribution of the repeat units</td>
<td>• Process conditions</td>
</tr>
<tr>
<td>• Presence of ionic groups</td>
<td>• Thermic treatments</td>
</tr>
<tr>
<td>• Presence of unwanted units or chain</td>
<td>• Sterilization method</td>
</tr>
<tr>
<td>defects</td>
<td>• Environment</td>
</tr>
<tr>
<td>• Water permeability</td>
<td>• pH, ionic force and temperature of the</td>
</tr>
<tr>
<td>• Configurational structure</td>
<td>degradation means</td>
</tr>
<tr>
<td>• Polydispersity</td>
<td>• Adsorbed and absorbed compounds</td>
</tr>
<tr>
<td>• Morphology and degree of crystallinity</td>
<td>• Degradation mechanism</td>
</tr>
<tr>
<td>• Presence of microstructures and</td>
<td></td>
</tr>
<tr>
<td>residual stress</td>
<td></td>
</tr>
<tr>
<td>• Porosity and quality of the matrix</td>
<td></td>
</tr>
<tr>
<td>and surface</td>
<td></td>
</tr>
</tbody>
</table>

Polymers used in tissue engineering can be natural, synthetic or a combination of both. There are many
advantages and disadvantages for both synthetic and natural polymers that are summarized in Table 2.3.1-2.

Table 2.3.1-2. Summary of the most common advantages and disadvantages of natural and synthetic polymers.

<table>
<thead>
<tr>
<th>POLYMER</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NATURAL</td>
<td>• Possess intrinsic biological activity</td>
<td>• Limited control of parameters</td>
</tr>
<tr>
<td></td>
<td>• Enzymatically degradable</td>
<td>• Source-related contamination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Potential for immunological responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Variation in degradation rates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Inferior mechanical properties</td>
</tr>
<tr>
<td>SYNTHETIC</td>
<td>• Easily synthetized with controlled physical properties</td>
<td>• Lack of intrinsic biological activity</td>
</tr>
</tbody>
</table>
2.3.2. Natural biopolymers

Natural polymers get the advantage to have specific cellular interactions called “cellular recognition”, but their availability is limited as they come from human, plant and animal tissues. Natural polymers include alginate, elastin, collagens, fibrins, proteins, gluten, albumin, hyaluronic acid, starch, cellulose, fibroin, chitosan, elsinan, scleroglucan, galactan, curdlan, pectin, gellan, emulsan, dextran, levan, heparin, silk, pullulan, chondroitin 6-sulfate, polyhydroxyalkanoates, etc. These materials are highly attractive thanks to their biocompatibility, bioactivity, ease of processing and remodeling, capability to present receptor-binding ligands to cells [47]. On the other side, natural polymers can be responsible for immunogenic responses and diseases transmission, they require high costs of manufacturing and have low versatility in producing scaffolds with specific characteristics [48, 49].

2.3.3. Synthetic biopolymers

Synthetic materials can be industrially produced on the large scale and can be processed in a three-dimensional matrix with various techniques where main structure, mechanical properties and degradation rate can be controlled. They have a known composition so that they can be designed in order to minimize their immunogenicity and adjust specific parameters during the synthetic process.
Synthetic polymers with different properties can be mixed, by combining their characteristics. The main disadvantage consists of the lack of specific signals for the cellular recognition. Those polymers are largely divided into two categories: biodegradable and non-biodegradable. Some synthetic non-biodegradable polymers are polyhydroxyethyemethacrylate (PHEMA), poly(N-isopropylacrylamide) (PNIPAAm) and polyvinyl alcohol (PVA). Some biodegradable polymers include the family of poly(α-hydroxy esters) such as polylactide (PLA) and polyglycolide (PGA), its copolymer polyanhydride, poly(lactide-co-glycolide) (PLGA), poly(propylene fumarate), polyphosphazene, polydioxanone (PDO), polycyanoacrylate, poly(ε-caprolactone) (PCL), and biodegradable polyurethanes. In tissue engineering, synthetic biodegradable polymers are preferred. They are used to create temporary scaffolds that work as mechanical and biochemical support by allowing the formation of new healthy tissue and minimizing the chronic reaction to a foreign body. Due to the minimal patient-to-patient and site-to-site variations, hydrolytically degradable polymers are mostly favored as implants instead of enzymatically degradable polymers [50].

2.3.4. Poly-ε-caprolactone

Poly-ε-caprolactone (PCL) is a semi crystalline synthetic biodegradable aliphatic polyester that can be obtained by ring-opening polymerization (ROP) of the correspondent
cyclic ester, ε-Caprolactone in the presence of a catalyst (figure 2.3.4). The repeat unit of the PCL is made of five apolar methylene groups and an ester group that is relatively polar.

![Diagram of polymerization of ε-caprolactone](image)

Figure 2.3.4. Polymerization of ε-caprolactone by opening of the ring [51].

This structure makes PCL similar to polyolefins because of the presence of methylene groups, while ester aliphatic bonds promote polymer degradation, as they are unstable to the hydrolysis. Since the homopolymer has long-term degradation properties (>2 years to lose total mass), copolymers have been synthetized to accelerate the absorption rate. PCL is known for its high thermal stability, good degree of crystallinity (~50%), extremely low melting point and glass transition temperature (T_m~60°C, T_g~60°C) [52]. Thanks to its elastomeric properties, high elongation and good biocompatibility PCL is of great interest. In Table
2.3.4 the most attractive properties are reported. PCL is largely used in biomedical applications for resorbable sutures, controlled release systems of drugs and substitution/regeneration of tissues [48].

Table 2.3.4. Summary of few attractive properties of PCL.

<table>
<thead>
<tr>
<th></th>
<th>$T_m$ (°C)</th>
<th>$T_g$ (°C)</th>
<th>Modulus (GPa)</th>
<th>Elongation (%)</th>
<th>Degradation Time (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>58-63</td>
<td>60-65</td>
<td>0.4</td>
<td>300-500</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

Some qualities that make PCL attractive for biomedical use are the ability to be processed at low temperatures, non-toxic degradation products, enhanced solubility in organic solvents and slow rate of degradation. Because of its great mechanical properties and the ability to blend easily, PCL was copolymerized in many works with various polymers. It was blended with starch in order to limit production costs and also to increase mechanical strength and promote cell growth with the presence of a natural biopolymer [53]. Other studies have shown that, PCL is able to increase the ductility of PLA when blended together. PCL mixed with collagen in composite films has been used to develop promising skin grafts for burned victims. The degradation
process was monitored \textit{in vitro} demonstrating that the presence of PCL provided the necessary stability, higher cell growth and adhesion [54].

2.4. \textbf{Essential scaffold properties}

Scaffold plays a fundamental role in tissue engineering applications. Its main function is to provide an appropriate substrate that promotes adhesion, growth, proliferation, differentiation and migration for both seeded cells and those on the surrounding tissue. Scaffolds should be able to offer the appropriate physical and chemical characteristics to guarantee proper tissue growth: be biocompatible and biodegradable, be easily processed into the desired shape, provide necessary mechanical strength and porosity, have high surface/volume ratio, supply cell-adhesion substrate, stimulate cellular response, etc. [47, 55]. Therefore it is important for scaffolds to fulfill some prerequisite:

- Presence of high surface/volume ratio to facilitate cells attachment in a reproducible macro-/microscopic structure.
- The scaffold should have proper support and mechanical properties for the success of the implantation. In particular it should mimic the natural tissue behavior as close as it can by showing similar flexibility to the surrounding environment and it
should be able to support the mechanical load on the damaged tissue while it repairs.

- The scaffold should have the right porosity that allows vascularization, cell ingrowth and metabolite transport.
- The scaffold material should be biocompatible, able to stimulate adequate responses from the host.
- The material should be biodegradable, with degradation products easy to remove from the body and non-toxic.
- Once the tissue is repaired, the scaffolds should disappear. It means that the degradation rate should be tailored in order to match the rate of tissue regeneration.

Other important parameters to consider are topography and surface energy since the surface is the first site of contact for cells. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, whereas the scaffold chemistry may have an important role in cell attachment and differentiation. A good porosity allows cellular attachment, migration and an adequate vascularization. On the other hand, the scaffold will lose the mechanical strength and will not be able to support cellular growth if there’s too much porosity [56].

Another important aspect to take into account is the surface coating. It has been demonstrated the great advantage in the inclusion of natural polymers into synthetic ones; natural polymers and growth factors together support cell
growth without cytotoxic effects. Healing and tissue regeneration can be stimulated also by including biological factors into the scaffolds: it can be growth factors or native ECM proteins. Within the fabrication process by ensuring 3D shape, sufficient mechanical and tensile strength as well as appropriate porosity and pore size distribution it will be possible to develop promising devices and alternatives in medicine regeneration.

2.5. Culture of cells for tissue engineering

Common treatment methods to injured or diseased tissues or organs have been including allografts, autografts and artificial prostheses until the introduction of tissue engineering. Tissue engineering is based on the production of tissue substitutes, which are able to recover the physiological functions and features of natural tissues in vivo. Tissue constructs are obtained mostly by growing isolated cells into biopolymer scaffolds by using different in vitro tissue cultures.

2.5.1. Primary and outgrowth cultures

To begin with, cells are isolated from a donor: a small fraction of tissue adheres to the growth substrate either
spontaneously or thanks to the aid of mechanical means, plasma clot or ECM constituent that usually induce an outgrowth of cells. Cells explanted first are called primary explant, cells that come to migrate out are known as outgrowth. After mechanical or enzymatic disaggregation, outgrowth cells are selected because of their ability to migrate from the explant and then adhere to a solid substrate by giving rise to a monolayer as cell suspension. Cells on the monolayer are capable of proliferation and, if sub-cultured, they may produce a new cell line. Usually disaggregated outgrowth cells generate larger cultures more quickly than primary explant [57].

2.5.2. Proliferation and differentiation

In general differentiating cells composing a tissue do not have the tendency to proliferate, unless selective conditions are used: satisfactory primary culture may be provided, which may be propagated as stem cells or mature ones toward differentiation. Thus, they are not able to form primary cultures if their differentiated status is not preserved and their attachment is not promoted someway [57, 58]. The largest potentially compartment of proliferating cells in a primary culture comes from the precursor special compartment of a tissue, like the fibroblasts of the dermis or the basal epithelial layer of the epidermis. Hence it is clear that it is essential to isolate the correct population of cells but also the proper conditions must be selected so that
cells are maintained at the appropriate stage with the capacity of proliferation whether population expansion is needed. In early culture of fibroblasts, for instance, the proliferative precursor phenotype was maintained by including serum, which contained growth factors like platelet-derived growth factor (PDGF) [59]. On the contrary, serum growth factors like transforming growth factor β (TGF-β) inhibited proliferation but promoted differentiation [60]. Even though, undifferentiated precursors may be the best option for expansion in vitro, the main requirement for transplantation is for cells to be able or have the potential to differentiate. Consequently two are the factors to be considered for both expansion and differentiation. Basically it can be affirmed that for differentiation a specific medium is required depending on the cell type, integrated with factors (retinoids, hydrocortisone, etc.) that promote differentiation, and planar-polar compounds (sodium butyrate, etc.). Moreover, it is important to determine the correct cell-to-matrix interaction, cell-to-cell interaction and cellular polarity, usually by using organotypic cultures. Whether extensive tissue regeneration is performed, the graft is asked to be totally (or almost totally) differentiated. In the case of stem cell transplantation, cell culture needs to expand a precursor cell type and then the implantation process will induce differentiation [61].
2.5.3. Organotypic and histotypic cultures

An attractive approach for tissue engineering is the use of organotypic cultures that is the recombination of specific cell types to create new cell lines in order to expand the cell stock. Through this approach, it is possible to growth tissues for studies based on cell-to-cell and cell-to-matrix interactions and for in vivo implantation. The fidelity of the neoconstruct depends on the presence of all the necessary cell types in the tissue in vivo and, above all, the capability of culturing and recombining those cells in the right proportion to the correct matrix and features [62]. Among the big variety of methods to recombine cells for tissue simulation, it can be found in allowing cells to multilayer by perfusing a monolayer [63], more complex perfused membrane [64] or capillary beds [65]. Those techniques of recombination give rise to histotypic cultures with the aim to achieve the natural cells density, cell interactions and matrix generation. By using specific media, cloning or physical separation methods, it is possible to isolate purified cell populations from primary culture or disaggregated tissue. Purified cells can be recombined to form organotypic cultures.
2.6. Microfabrication in biology and medicine

Recently, microfabrication approach has been attractive for biological and medical fields by offering several opportunities to study chemical, physical and biological processes at the cellular and molecular scale and, consequently designing devices characterized by complex features and functionalities able to naturally interact with biological systems. A great advantage is represented by the micro-scale since it is possible to fabricate implantable, portable or even injectable systems. Moreover, the use of these devices reduces the time to diagnose patient’s conditions allowing a better management of the decisions related to the patient itself, obtaining improved results and significantly reducing the cost of care. Microfabrication techniques conferred to scientific and medical world the possibility to improve and expand the applications of devices already created, thanks to the miniaturization and the enhanced control of size, morphology, topology and functionality. These methods are promising tools for the realization of new approaches to the study of molecules, cells and tissues since they enable the fabrication at cellular and sub-cellular levels; new treatment alternatives and pathological mechanisms can be investigated. Many techniques have been developed for the creation of micro-scale tools, some of them deriving from the field of semiconductors. The desired structure is obtained by following a sequence of microfabrication process steps. It
can be realized on either the surface of the substrate material [66] or within the bulk of the substrate material [67]; these two processes are defined surface micromachining and bulk micromachining respectively. In most cases, the desired device is built by using both types of micromachining. In both cases, the most important microfabrication methods employed are photolithography, soft lithography, film deposition, etching and bonding.

2.6.1. Photolithography

Photolithography is one of the most used microfabrication methods and consists of the transfer of a specific pattern onto the surface of a substrate by means of the selective exposure of the material that is sensitive to ultraviolet (UV) light [68, 69]. The process is summarized in Figure 2.6.1. Firstly, the desired pattern is drawn with a computer-assisted design (CAD) program and is then transferred onto a photomask, that is a glass plate with the surface made of photodefinable opaque regions (usually chrome) in the desired pattern. Then, a substrate material (silicon, glass, etc.) is coated with photoresist, a photosensitive organic polymer. The pattern transfer starts when the mask is placed on top of the resist and substrate and the whole assembly is irradiated with UV light: photoresist sections that are not covered by the opaque regions of the mask are exposed. According to the kind of the photoresist used, two reactions are possible. When positive resist is exposed to
light, the illuminated regions of photoresist can be removed since they become more soluble in developing solutions. On the contrary, when light illuminates a negative photoresist exposed areas become cross-linked and they are insoluble in developing solution: only those parts that are not exposed could be removed.

Figure 2.6.1. Photolithography process: a photomask made of opaque regions in the desired pattern is used to transfer a specific shape onto a light-sensitive photoresist that is selectively illuminated. After UV exposure, photoresist becomes more soluble, if it is positive, or cross-linked, if it is negative. Hence the appropriate pattern is generated upon developing.
After that, the resist survived to light exposure, works as protection for the underlying substrate from etching or from deposition of biomolecules. Once the final pattern is obtained, the residual resist usually can be removed by sonication in an organic solvent; this method could be inappropriate for systems containing biomolecules, so water-soluble photoresists could be used as alternative. With photolithography it is possible to obtain several pattern features and high resolution that depend on the characteristics of the photomask. The limitation represented by this technique is the need of clean room processing.

2.6.2. **Soft lithography**

Soft lithography technique is similar to photolithography. It uses a soft polymeric stamp that is reusable. It consists of three main processes: micro-contact printing, stencil patterning and microfluidic patterning. The three steps are represented in Figure 2.6.2. Micro-contact printing is the realization of a stamp with the designed pattern through a polymer master. Self-assembled monolayers are formed by placing molecules to be transferred on the stamp surface and then printed on the receiving substrate surface [70]. Stencil patterning allows the creation of templates used to prevent the cover of the master template from polymer: a polymer model with holes in the pattern of the master is obtained. Another way to avoid polymer from covering the
master features, is to put plates between the master and the polymer [69].

Figure 2.6.2. Schematic representation of the three soft lithography techniques: micro-contact printing, stencil patterning and microfluidic patterning. Microstructure replica is produced from a polymer mold obtained from other fabrication methods.
Microfluidic patterning is based on the use of polymer mold to create micro-channels within the substrate, which are able to pattern fluid materials onto the substrate itself [69].

**2.6.3. Film deposition**

The application or growth of films or layer of materials on the surface of microstructures, is used for a variety of aims in microfabrication: films may work as sacrificial or masking layers, electrical devices, structural or functional materials, etc. Physical or chemical reactions processes can form them.

**2.6.4. Etching**

The purpose of the etching process is the realization of particular topographical features on the substrate surface thanks to chemical and/or physical removal of material. There are two mechanisms used for etching: wet etching via liquid chemicals and dry etching via gas-phase chemistry. In both cases, etching can be isotropic if it occurs equally in all directions or anisotropic if it occurs in specified directions [69]. In Figure 2.6.4 different etching phases can be observed: isotropic etching is shown in Figure 2.6.4A; it proceeds either laterally and in depth, resulting in a rounded etch profile. Figures 2.6.4B and 2.6.4C, show
anisotropic etching that occurs in only one direction usually increasing the depth.

![Diagram of different etching profiles](image)

Figure 2.6.4. Overview of different etching profiles: (A) isotropic etching; (B) dry anisotropic etching and (C) wet anisotropic etching [71].

In the last two Figures also the difference between wet and dry etching can be observed: in the former case etching results in a flat profile, in the latter the result is cavities with inclined sidewalls.

### 2.6.5. Bonding

Some structure can be obtained after the formation of reversible or irreversible bonding between microstructures.
Depending on the material of interest, there are different bonding techniques available. Some example could be the use of heat for bonding of polymers, overcoming the glass transition temperature and applying the right pressure to seal the structures, by means of ultrasonic welding or laser welding [72]. A silicon substrate and a non-pure glass film can undergo irreversible anodic bonding by applying high pressure, an electric field and temperatures around 400°C [68]. Also adhesives can be used to bind two materials, even though it could affect the characteristics of the system.

### 2.6.6. Advantages of microfabrication

Among the advantages offered by microfabricated devices the capability to control features even on the nanometer scale is a great one, in addition to the inclusion of electronic elements via well-developed semiconductor techniques and the miniaturization of systems already fabricated for the investigation of cellular and molecular processes. Moreover, the knowledge achieved about microfabrication leads to the combination of environmentally sensitive polymers, nanoparticles and micro-electromechanical systems (MEMS) elements in order to design controlled release systems. The advantages conferred by microfabrication are summarized as follow:

- Small size devices have the advantage to be portable and occupy a limited space. Instead of being in
centralized laboratories, they are hand-held or fit into small systems.

- High surface-area-to-volume-ratio: the more the device is miniaturized, the more the surface effects dominate the volume effects that lead to significant improved physical results.

- The correlation between microfabrication and the well-established semiconductor fabrication makes possible the integration of electronic components with microfabricated devices. This can give rise to sensors or some tool that are able to record and manage bio-signals, thus amplifying them and providing appropriate stimuli [73, 74].

- Often small-scale-size and enhanced surface to volume mean together high throughput and faster analysis.

- Samples having lower volume provide some benefit in terms of costs since reagent volumes and waste disposal are reduced. Moreover, they are pretty useful for drug delivery applications and some medical diagnostic: small volumes devices can enter small sites, allowing easier detection and better monitoring of cells physiology and single-cell analysis [75].

- A lot of microfabrication processes can be easily employed on a multitude of identical devices without caring of the variations that could involve an individually constructed object. This is called batch processing.
• Another important aspect is constrained geometry that allows the confinement of molecules or mechanical forces. In particular confining of molecules is a great advantage since it prevents diffusion out of the volume and enables higher molecule’s local concentration.
• Geometrical control is a very important factor for microfabricated structures. Photolithography technique allows the realization of different patterns characterized by features (even with the same structure) with a large variety of geometries in the same space. Therefore it is possible a precise control of the spatial organization.

2.6.7. Disadvantages of microfabrication
As microfabrication is a means to improve or supplement well-established technologies, there are reasons why some structure cannot be microfabricated:

• If the systems to be produced is too complex, microfabrication processes could require long times.
• Depending on the application, there could be lack of appropriate structures or materials availability.
• The enhanced surface-area-to-volume-ratio could lead to higher surface adsorption of biomolecules.
• The use of small volumes samples could make sample evaporation problems (very small quantity of fluid can evaporate in seconds). In addition, the detection of
molecules in dilute solution could be more difficult, since the smaller the number of molecules, the smaller the cube of the volume for a given solute concentration.

- If the number of devices required is small and the dimensions are reasonable (>100µm), it is possible, and sometimes faster, to build them conventionally.
Chapter 3

Design and fabrication of the device

In this chapter, the two fabrication approaches employed to accomplish the purpose of the current work are presented. The entire manufacturing micro-process consists of the use of optical lithography techniques for the generation of Si masters. Once the master is available, the majority of the molding fabrication procedures can be performed outside the clean room. The material used and each step of the fabrication methods is described with their basic functioning. Schematic summary of the scaffolds production process is shown: microfabrication steps in Figure 3.1 and micro-molding steps in Figure 3.2. Nano-patterned pillared PCL devices for 3D cell growth were realized by lithographic and molding techniques within a negative Si master. Cylindrical pillars with 10µm in height, 10µm in diameter were arranged in a hexagonal lattice with 30µm periodicity. The substrates show a combination of the patterned length scales. Specifically, they were designed in micro-scale while the sidewall was nanostructured; this gives rise to a good spatial modulation in the z direction.
Figure 3.1. Schematic representation of the microfabrication processes of biocompatible 3D PCL devices. (a) Si wafer used for production of the negative molds. (b) Si wafer coated by positive tone resist S1813. (c) Representation of the hexagonal pattern transferred from a photomask after optical lithography process. (d) With DRIE process, Si wafer was digged in order to obtain cylindrical holes with 10µm diameter, 10µm height and 30µm periodicity. (e) Final Si masters obtained after removal of the residual resist.
Figure 3.2. Schematic representation of the micro-molding processes of biocompatible 3D PCL devices. (a) Front and (b) perspective view of the first part of the micro-molding approach: PCL pellets placed on a slide are let melting after heat is provided. (c) Front and (d) perspective view of the second part of the micro-molding approach: after decreasing of the temperature and providing an adequate force, the desired scaffold is obtained. (e) Representation of the 3D PCL nanostructured scaffold after separation from the Si master.
3.1. Photolithography approach

Photolithography technique involves a sequence of steps executed according to a given order to produce the final device.

3.1.1. Spin coating of Si wafers

![Spin-coater](image)

Figure 3.1.1-1. Spin-coater used for the coating of Si wafers by positive photoresist.
4 inch, P-doped Si wafers have been used as substrates for the fabrication of the mold, characterized by resistivity 1-10 Ω/cm and 525µm thickness. To begin with, the wafers have been cleaned from any contaminant, by following a precise procedure: first, it was wet cleaned with acetone (Sigma-Aldrich) and isopropanol (Sigma-Aldrich), then rinsed several time in deionized water and dried with N₂.

Figure 3.1.1-2. Hot plate used for heating of the wafers in order to cure the light-sensitive resist.
In order for microlithography to be employed, a spin coater (Figure 3.1.1-1) was used so that a thin layer of positive tone photoresist (Microposit S1813, Dow Electronic Materials) was spin-coated onto the prepared Si substrates. A solution of S1813 was placed on the surface of a Si wafer by means of a plastic pipette. The spin-coating process was realized at 4000 rpm for 60 sec and the liquid resist solution spanned uniformly the entire surface. After that, the layer of S1813 covering the Si wafer surface underwent a bake process at a temperature of 95°C for 5 min; typical hot plate used during this step is shown in Figure 3.1.1-2.

3.1.2. Optical lithography

As already said, optical lithography is a process employed to selectively remove portions of a thin film or a substrate. UV light was used to transfer a specific geometrical pattern from a photomask to the photoresist spin coated onto the Si substrates. The mask used for the photolithography process (Mask Aligner MA 45, Karl Suss shown in Figure 3.1.2-1) was created by using Electron Beam Lithography (EBL, Crestec CABL 9000 shown in Figure 3.1.2-2A). Photoresist spin coated Si wafer was aligned with the photomask and, after exposure to the light, standard photolithography techniques allowed a regular pattern of disks with hexagonal periodicity (Figure 3.1.2-2B) to be transferred on the layer of positive tone resist. Then fragments of photoresist were dissolved, by immersing the substrate into
MF-319 developer for 1 min. The duration of the development process is important as the longer the development, the more the dissolution extends.

Figure 3.1.2-1. Mask aligner used for optical lithography processes.
Figure 3.1.2-2. (A) EBL used for the fabrication of the photomask. (B) Draft of the geometrical pattern to be transferred on the photoresist coating the Si wafers.
3.1.3. Deep Reactive Ion Etching process

After photolithography, the following step was Deep Reactive Ion Etching (DRIE, ICP-RIE, Surface Technology Systems, STS shown in Figure 3.1.3) of the silicon, in particular it was used the Bosch process also known as pulsed or time-multiplexed etching. The pattern of disks on the resist layer served as mask for the process itself.

![DRIE system used for the etching process.](image)
The procedure consisted of repeated cycle characterized by 1) C₄F₈ – chemically inert passivation layer deposition and 2) SF₆ – based anisotropic Si etching. At the end of each cycle, a refractory period of reactor cleaning and gas evacuation was performed. After that, the residual photoresist was removed by means of sonication in acetone (65°C) and the wafers were deeply cleaned with a piranha solution. Therefore, after processing of bulk Si wafers, negative Si molds were obtained resulting in periodic hexagonal lattice of cylindrical holes with nanostructured vertical sidewalls, 10µm depth, 10µm diameter and 20µm spacing between them. Finally, Si substrates were cut in a number of separated pieces by a diamond tip and subjected to standard cleaning procedure: washed in acetone, rinsed in isopropanol and dried with N₂.

3.2. Micro-molding approach
Micro-molding technique involves essentially two main procedures: steps leading to the melting of the polymer and after imprinting and cooling of PCL, the final scaffold is obtained.
3.2.1. Hot press

3D PCL micro-structured nano-patterned scaffolds were fabricated within micro-molding method as follows. A glass slide, that was previously cleaned with acetone and rinsed in deionized water, was positioned on the lower plate of a hot press (Presstronic, p/o/weber Lab press technology shown in Figure 3.2.1). PCL (Sigma Aldrich, $M_n = 70000-90000$, $T_{\text{melt}} = 60^\circ\text{C}$) in pellets was then placed onto the glass support and let it melt at $T = 133^\circ\text{C}$.

Figure 3.2.1. Hot press used during the micro-molding phase.
Once $T = 133^\circ$C was reached, the negative Si master was put in contact with the melted PCL by avoiding the possible formation of air bubbles at the polymer-Si interface. After that, another glass slide was placed on top of the Si mold in order to prevent the breaking of the mold itself during imprinting process. The first part of the molding proceeding was performed when the temperature was set to be $T = 68^\circ$C and a force $F \sim 1.60\text{kN}$ was manually applied. The provided force immediately decreased of $\sim 0.05\text{kN}$ and stabilized. The second part was about cooling of the PCL, followed by solidification of the polymer until room temperature ($\sim 25^\circ$C) was reached. The whole process lasted $\sim 8\text{ min}$ and in the last 3 min the force underwent a further decrease of $\sim 0.1\text{kN}$. The desired scaffold was obtained after removing of the glass supports, while master detachment from PCL was performed by applying a small pull after being left in ice for $\sim 15\text{ min}$. The result was positive PCL structures texturized in a periodic hexagonal lattice of micro- and nanostructured pillars.
Chapter 4

Cells seeding

In this chapter, the preparation process of the scaffolds and consequent cells plating is described. The fabricated bio-devices are tested by evaluating the behavior of two different cell lines: human Neural Stem Cells (hNSC) and NIH/3T3 mouse embryonic fibroblast cells.

4.1. Substrates preparation for human Neural Stem Cells

To begin with, PCL substrates were sterilized: immersed in 99.9% ethanol (Sigma-Aldrich) for 3 sec and washed with sterile deionized water. After being dried within a vacuum system, samples were sterilized further by UV irradiation for 90 min. Afterwards scaffolds were put in the culture medium (KnockOutTM DMEM/F-12, Life Technologies) keeping them in an incubator for 48 hours (37°C, 5% CO₂, 95% humidity). Once removed from the medium, the substrates were immersed in a 0.1mg/ml water solution of poly-D-lysine (PDL, Sigma-Aldrich) overnight. Before moving
to the seeding of cells, PCL scaffolds were washed in sterile H₂O to remove PDL and then let dry. 8x10⁴ hNSC (GIBCO® H9 hESC-Derived, Life Technologies) were plated on PCL substrates coated with PDL through “drop seeding” method. 150µL drop of plating medium (StemPro NSC SFM complete medium, Life Technologies) was seeded on each device, by assuming a quasi-spherical shape. The high relative humidity of cell culture incubator helped to avoid medium evaporation and reduction of the diameter of the drop. After 4 hours, fresh medium integrated with 2% StemPro Neural Supplement was supplied. Equal number of hNSC was also seeded on PDL-coated polystyrene dishes that worked as controls.

4.2. Substrate preparation for NIH/3T3 mouse embryonic fibroblasts

Fibroblast cells were obtained from the American Type Culture Collection (ATCC). They were placed in Dulbecco’s Modified Eagle’s Medium (DMEM) and cultured with high glucose. 10% (v/v) fetal calf serum, penicillin G (100 U/ml) and streptomycin sulfate (100µg/ml) were added. After sterilization, PCL devices were treated for 48 hours with PDL hydrobromide (P6407, Sigma-Aldrich) and DMEM medium complete in a 1:1 (v/v) ratio respectively. Cells were grown in a CO₂ atmosphere with 5% humidity at a
temperature of 37°C. Once substrates were rinsed with sterile H₂O and dried, 4x10⁴ fibroblast cells were seeded on them and let grow for 24 hours.

### 4.3. Fibroblasts Scanning Electron Microscopy

After 24 hours growth, fibroblasts were fixed in 1.2% (v/v) gluteraldehyde (Sigma-Aldrich) and 0.1 M sodium cacodylate (Sigma-Aldrich) solution for 1 hour at temperature of 4°C. Then cells were widely rinsed in 0.1 M pH 7.4 sodium cacodylate buffer and post-fixed in 1% (v/v) osmium tetroxide and 0.1 M sodium cacodylate solution for 1 hour. A number of washes in Milli-Q water of the fixed samples were followed by rinses for 5 min in rising concentrations of filtered ice-cold ethanol (30%, 50%, 70%, 80%, 90% and 96% v/v) and two washes in filtered ice-cold 100% (v/v) ethanol for 15 min. After graded dehydration with ethanol, hexamethyl disilazane (Sigma-Aldrich) was used for a gradual replacement and evaporated overnight in a fume hood. Finally substrates were coated with 10nm gold/palladium in a sputter coater and, once placed on SEM stubs, they were observed with a variable pressure SEM (JEOL JSM-6490LA).
4.4. Human Neural Stem Cells

**Immunofluorescence Characterization**

Human Neural Stem Cells were fixed with 2% pH 7.4 paraformaldehyde (PFA) and rinsed for 3 times in phosphate buffered saline (PBS) 1x. Substrates were then permeabilized with 0.3% Triton X-100 for 10 min and blocked with 10% (w/v) Bovine Serum Albumin (BSA) + 0.1% Tween20 for 1 hour. The blocker solution was supplemented with Polyclonal Rabbit anti-GFAP (Glial Fibrillary Acid Protein) primary antibody (ab7779, Abcam) in order to mark astrocytes and incubated overnight at 4°C. The day after, Alexa Fluor 568 F(ab')2 Fragment of Goat Anti-Rabbit IgG (H+L) secondary antibody (A21069, Invitrogen) was added after two rinses in PBS 1x. Moreover, cells were stained with 1mg/ml DAPI (4’, 6-diamidino-2-phenylindole) for 20 min (1:500) in order to mark nuclei. Leica TCS SP2 Confocal Microscope equipped with 63.0x1.4 A.N. and 40.0x1.25 A.N. objectives was utilized to get the images.

4.5. NIH/3T3 Mouse Embryonic Fibroblasts

**Immunofluorescence Characterization**

NIH/3T3 mouse embryonic fibroblast cells were fixed at room temperature with 4% PFA for 60 min. They were then
permeabilized with 0.5% Triton X-100 for 10 min and blocked with 1% (w/v) BSA in PBS. At this stage, samples were stained with antibody against Vinculin (Abcam ab18058) for 60 min and detected with chicken anti-mouse Alexa Fluor 488 (Invitrogen) secondary antibody. Moreover, fibroblasts were incubated for 30 min with Alexa Fluor 546 phalloidin at a temperature of 4°C in order to be stained with F-Actin. Pictures of the results were obtained from an upright Leica TCS SP5 AOBS TANDEM Confocal Microscope equipped with 40X/0.80 APO L W UVI objective.
Chapter 5

Results

Microfabrication (Figure 3.1) and micro-molding (Figure 3.2) approaches have been successfully used for the creation of positive biocompatible 3D pillared substrates from a negative Si mold, as semi-crystalline PCL is characterized by long time degradation, good solubility and low melting point (59-64°C). Those fabrication techniques have shown the great advantage to be simple and flexible as well as low cost.

5.1. SEM characterization of the scaffolds

SEM studies were performed after coating of the substrates with 10nm gold in a sputter coater by using a Dual-beam SEM/FIB (FEI Novalab 600i System) showed in Figure 5.1-1. The morphology of the device was evaluated: PCL pillars with cylindrical shape characterized by hexagonal periodicity, diameter of 10µm, height 10µm and spacing of 20µm (Figure 5.1-2a, b). The design of the pillars surface was in micro-scale while the sidewall of the pillars presented nano-features with a regular pattern of grooves (Figure 5.1-2c).
The combination of these two different length scales allows a well-organized spatial modulation in z direction and introduces the 3D topology that makes the device mimicking properly the natural environment for an appropriate cell distribution. In fact, the pillared surface gives rise to an interconnected structure with high surface

Figure 5.1-1. Dual-beam SEM/FIB used to perform SEM analysis.
area promoting cell ingrowth and consequent adhesion and expansion.

Figure 5.1-2. SEM characterization of the device. (a) Low and (b) high-magnification of the of the upper view and side view of the scaffold respectively. (c) Detail of the nano-patterned texture on the side of the pillars. 20µm bars.
5.2. **SEM characterization of NIH/3T3 mouse embryonic fibroblasts on 3D pillared scaffolds**

After seeding of fibroblasts, the *in vitro* development of the drop-plated cells on the biocompatible substrates has been characterized by SEM in order to evaluate the interactions of the cells with the microfabricated pillars. Highly packed cells (Figure 5.2a) and some patches of cells in groups (Figure 5.2c) have been observed.

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Figure 5.2. SEM pictures of NIH/3T3 mouse embryonic fibroblasts seeded on the PCL nano-structured pillared substrates. (a, c) Small filopodial processes (arrows) and (b) pseudopodia-like extensions (asterisk) were produced by showing good adhesion to the base of the pillars and bio-sensing the microdevice. 10µm bars.
Nevertheless, there is still a huge number of isolated cells that are shown to effectively adhere and interact with the pillars (Figure 5.2b). Micro and nano-characteristics of the PCL scaffolds had the capability to spatially control and direct cells to grow healthy, by forming a suspended 3D network. Fibroblasts, singularly or grouped in large bundles, adhered to the plateaus of the pillars, then extended and wrapped the grooved surfaces of adjacent pillars. This organization is an evidence of the specific pillars arrangement, as can be seen in Figure 5.2a, b. Moreover, after 24 hours, fibroblasts produced pseudopodia-like extensions (white asterisk), which anchored the base of the pillars, and small filopodial processes (white arrows), which interacted with the microstructure of the bio-substrate (Figure 5.2b and 5.2a, c respectively). The presence of filopodia and pseudopodia-like represents the good adhesion and spreading of cells that consequently shows the good cytocompatibility of the devices.

5.3. Human Neural Stem Cells

Immunofluorescence analysis

Human Neural Stem Cells were also plated on the 3D nanostructured PCL substrates.
To better characterize the biocompatibility of the scaffolds with the growth and proliferation of the hNSC, an immunocytochemical analysis was performed in order to study the viability of the cultures developed on nanopatterned PCL pillars (Figure 5.3-1) compared to cultures developed on standard polystyrene plates used as controls (Figure 5.3-2) at 6 days in vitro (DIV).

Figure 5.3-1. Double staining confocal microscopy analysis of hNSC cultures at 6 DIV plated onto polystyrene substrates. Red: GFAP; blue: DAPI. 20µm bars.*
Immunofluorescence analysis involved the usage of GFAP antibody, so that in vitro differentiation of hNSC into astrocytes (Figure 5.3-1a, c and Figure 5.3-2a, b, e, f) has been evaluated (red signal), and DAPI in order to highlight (blue signal) the nuclei of the cells (Figure 5.3-1b and Figure 5.3-2c, d). Though cultures grown on polystyrene dishes
Figure 5.3-1 showed morphology analogous to cells cultured on PCL substrates (Figure 5.3-2), it can be clearly seen that neurons distribution on the 3D system results in a more natural alike arrangement, giving rise to the formation of denser bundles of cells which span well between pillars. Under the designed conditions, cells grew in a viable 3D neural network. In addition, the large amount of free space among pillars of the proposed PCL nanostructured devices facilitates efficient gas and nutrients exchanges between cells.

5.4 NIH/3T3 mouse embryonic fibroblasts immunofluorescence analysis

Immunofluorescence analysis was performed to evaluate also the cytocompatibility of the fibroblasts (Figure 5.4). In order to highlight the great capability of adhesion and proliferation of fibroblast cells onto the nanostructured device, F-actin (red signal) and vinculin (green signal) proteins were stained. The expression and localization of these markers showed physiological growth and development of cells, making visible the formation of filopodia and lamellipodia-like structure that favored a strong adhesion at the base of the pillars. This is of crucial importance for cellular polarization and spreading.
Moreover, thanks to actin filaments, extensions of sheet-like and rod-like projections, cells were able to move through the cell-surface front of the substrates. The offered environmental conditions allowed fibroblasts to optimally proliferate on the device’s surface: topographical and biochemical information from the ECM were collected, cells grow healthy and disposed in a suspended natural-like arrangement.

Figure 5.4. Double staining confocal microscopy analysis of NIH/3T3 mouse embryonic fibroblasts cultures plated onto 3D nanostructured pillared PCL substrates. Red: F-actin; Green: vinculin. 20µm bars.**
Chapter 6

Summary

6.1. Conclusions

Nowadays it is well known that 3D polymeric scaffolds for cell culture technology are of increasing interest for tissue engineering and regenerative medicine. In fact, 2D culture systems such as flat plates made of polystyrene or glass, cause the cells to grow and expand in an unnatural way. It is important to consider all the features related to the structure or function of a specific tissue, so that a specific device with the desired mechanical properties, degradation rate, shape and porosity can be produced closely mimicking the natural environment. The importance in the optimization of the fabrication process is pointed out in the described work where a simple, economic and effective fabrication method is proposed by allowing the realization of 3D biocompatible devices that provide friendly physiological conditions for development and survival of cells. The micro-PCL pillars with nano-features on the sidewall, form a three dimensional system that enables cells to proliferate and
span by giving rise to a suspended natural-like network. In these environmental conditions cells can move and migrate thanks to the forces exerted on one another; in addition the particular pillars arrangement favors effective oxygen, nutrients and waste exchanges. Thus, the proposed 3D device constitutes a promising tool in tissue engineering and regenerative medicine and can be developed as a technological platform for exploiting the ability of some cell lines to assist the regeneration after injury.

6.2 Future perspectives

Despite the important results obtained, there is still a long way to go for making more precise future biocompatible devices for *in vitro* and *in vivo* applications. As the geometry has shown to be a fundamental aspect, it could be possible to manage cell adhesion and proliferation by modifying topographic features of the surface (for example the degree of roughness of the pillars sidewall of the presented devices). Cell differentiation applications could be checked by using different panels of antibodies. Employment of the appropriate cell type, especially among stem cells, could allow the handling of the differentiation process resulting in cells differentiating into diverse tissues and organs characterized by physiological and structural functionality.
References


biotechnology, 26(1), 39-47.
biotechnology, 22(2), 151-152.


[34] F. Grinnell. “Fibroblast biology in three-dimensional


[52] A. Sarac et al. “The ratio of crystallinity and


[60] H. L. Moses et al. “TGF-β stimulation and inhibition of


APPENDIX

Two papers have already been published before the submission of the present work:
