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FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

DESENVOLVIMENTO DE SUPERFÍCIE FACILITADORA DA ADESÃO CELULAR A DISPOSITIVOS BIOMÉDICOS COM UTILIZAÇÃO EM CIRURGIA MAXILO-FACIAL, PLÁSTICA E MEDICINA DENTÁRIA

IVANA DA COSTA TEIXEIRA

DISSERTAÇÃO DE MESTRADO APRESENTADA
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Presidente Professor Doutor José Alberto Peixoto Machado da Silva
Professor Associado do Departamento de Engenharia Eletrotécnica e de Computadores da Faculdade de Engenharia da U. Porto

Doutor Jorge Serafim Sobrado Marinho
Especialista na área de Estomatologia do IPO - Instituto Português de Oncologia do Porto

Doutora Christiane Laranjo Salgado
Investigadora Pós-Doutoramento do Departamento de Engenharia Metalúrgica e de Materiais da Faculdade de Engenharia da U. Porto

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Ivana da Costa Teixeira
Autor - Ivana da Costa Teixeira

Faculdade de Engenharia da Universidade do Porto

This dissertation was supervised by:

Doctor Christiane Laranjo Salgado (supervisor)

INEB - Instituto de Engenharia Biomédica

Faculdade de Engenharia da Universidade do Porto, Universidade do Porto

Professor Ana Reis (co-supervisor)

INEGI - Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial

Institution where this dissertation was performed:

INEB - Instituto de Engenharia Biomédica, Divisão de Biomateriais, Universidade do Porto, Porto, Portugal

Resumo

Na última década, os campos da engenharia de tecidos e medicina regenerativa têm emergido de modo a oferecer uma nova e entusiasmante alternativa para a reconstrução maxilofacial. Esta proporciona uma nova opção que visa complementar o tratamento existente para a reconstrução e a regeneração do complexo oral e craniofacial, que inclui os dentes, periodonto, ossos, tecidos moles (mucosa oral, conjuntiva e pele), glândulas salivares, articulação temporomandibular (osso e cartilagem), bem como vasos sanguíneos, músculos, tendões e nervos. Devido à complexicidade anatómica desta zona (osso, cartilagem, tecidos moles, nervos e vasos) a sua reconstrução envolve diversas áreas de especialização. Contudo, tanto para dentistas como para cirurgiões maxilofaciais, a reconstrução de defeitos na zona maxilofacial continua a ser um desafio. O sucesso clínico após colocação de um implante é principalmente influenciado pelas propriedades do biomaterial. Deste modo, o objectivo desta dissertação foi revestir discos de titânio com um polímero, a policaprolactona (PCL) a 7,5% e 10% (m/v) através da técnica de spin coating de modo a facilitar a adesão celular e melhorar a regeneração de tecido conjuntivo à superfície do material. Devido à hidrofobicidade característica deste polímero, algumas amostras foram, previamente, tratadas com solução alcalina (2N NaOH). A análise da morfologia (SEM) revelou que todas as amostras apresentaram um filme homogéneo, com a superfície dos discos completamente coberta com os esferulitos poliméricos. Após imersão das amostras em fluído corporal simulado (SBF) durante 28 dias, não foram reveladas drásticas alterações morfológicas ao material. A análise mecânica (AFM) mostrou um ligeiro aumento da rugosidade de superfície das amostras previamente imersas em NaOH. No entanto, não foi registada uma diferença significativa nas propriedades mecânicas das amotras (valor do módulo de Young inalterado). Foram efectuados diversos estudos *in vitro* utilizando fibroblastos L929 e fibroblastos dérmicos humanos neonatais (HDFn) a fim de avaliar a viabilidade celular nos filmes de policaprolactona (estudos de adesão, proliferação, morfologia e actividade metabólica). Todos os materiais apresentaram baixa citotoxicidade e boa proliferação celular. Observou-se a formação de densas camadas celulares sobre os diferentes tipos de materiais. Os fibroblastos L929 apresentaram uma morfologia normal e a sua confluência em cultura foi alcançada mais rapidamente do que as HDFn. Ambas as células foram capazes de produzir fibras de colagénio, o constituinte principal da sua matriz extracelular (ECM). Em conclusão, os discos de titânio revestidos com PCL mostraram biocompatibilidade satisfatória, aumentando o seu potencial para aplicações maxilofaciais.

Abstract

Within the past decade, the field of tissue engineering and regenerative medicine has emerged that offers a new and exciting alternative for maxillofacial reconstruction. It offers a new option to supplement existing treatment for reconstruction and regeneration of the oral and craniofacial complex, which includes the teeth, periodontium, bones, soft tissues (oral mucosa, conjunctiva, skin), salivary glands, and the temporomandibular joint (bone and cartilage), as well as blood vessels, muscles, tendons, and nerves. Due to this complex structure, a multi-specialty involvement in their management may be needed. However, for dentists and oral and maxillofacial surgeons, the reconstruction of maxillofacial defects in hard and soft tissues is an ongoing challenge. The successful clinical outcome of the implantation of biomaterials is strongly dependent on their material properties. In this work, the main goal was coated titanium discs with polycaprolactone at 7.5% (m/v) and 10% (m/v) by spin coating technique in order to improve cell adhesion and to enhance connective tissue regeneration. Due the PCL hydrophobic surface, some samples were previously immersed in 2N NaOH solution. Morphology analysis (SEM) revealed that all samples presented a homogeneous film with the polymeric spherulites completely covered the titanium surface with no drastic changes in its morphology after immersion in SBF (simulated body fluid) for 28 days. Mechanical analysis (AFM) showed a slight increase of roughness in samples after NaOH treatment. Nevertheless there was not significant difference in the mechanical properties (unaltered young's modulus value). Different cellular aspects, using fibroblasts L929 and Human Dermal Fibroblasts neonatal, were analyzed in order to know the cells viability during cell culture on PCL films: cell adhesion, proliferation, metabolic activity and morphology. All materials had low cytotoxicity, and higher cell proliferation. It was noticed the formation of a dense cellular multilayered cover on the materials. The L929 fibroblasts remained with their normal morphology and high cellular confluence in culture which was reached faster than HDFn cells. Both cells were able to produce collagen fibers, which is the primary constituent of their extra cellular matrix (ECM). In conclusion the titanium discs coated with PCL showed satisfactory biocompatibility, which enhances their potential for application in maxillofacial reconstruction.

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Contents

List of figures

List of tables

Abbreviations, acronyms and symbols

Acronyms and abbreviations list

AFM	Atomic Force Microscopy
CLSM	Confocal laser scanning microscopy
Cp Ti	Commercially pure titanium
DNA	Deoxyribonucleic Acid
EE	Ethinyl estradiol
EMT	Epithelial-Mesenchymal Transition
ePTFE	Expanded polytetrafluoroethylene
FBR	Foreign-body reaction
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HCl	Hydrochloric acid
INEGI	Instituto de Engenharia Mecânica e Gestão Industrial
LNG	Levonorgestrel
Max	Maximum
MEM	Minimum Essential Medium
MET	Mesenchymal-epithelial transition
Min	minute
Ml	mililiter

NaOH	Sodium Hydroxide
nm	nanometer
NO	Nitrogen Oxide
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PCL – PLLA	Polycaprolactone and Poly-lactide
PCL – DLLA	Polycaprolactone and D, L-lactide
PCL – TMC	Polycaprolactone and trimethylene carbonate
RFU	Relative fluorescence units
Rpm	Rotation per minute
SBF	Simulated Body Fluid
SEM	Scanning electron microscope
TCA	Tricarboxylic acid
TERM	Tissue Engineering and Regenerative Medicine
Ti	Titanium

Symbols list

α	Alpha
ß	Beta
s	Seconds
µl	Microliter
µm	micrometer
2D	two-dimensional
3D	three-dimensional
°C	Celsius

Chapter 1 - Introduction

Accidents and diseases lead to devastating tissue lost and organ failures which could represent a life threatening situation [1]. For dentists and oral-maxillofacial surgeons, the reconstruction of hard and soft tissues is an ongoing challenge due the complex array of bone, cartilage, soft tissue, nerves and vessels [2]. Even a minimal damage to these structures usually leads to noticeable deformities [3]. In order to overcome such damages, extensive studies in the field of regenerative medicine and tissue engineering are focused on restoring body function through implants as an alternative to current surgical techniques or approaches [3] [4]. The past half century has seen an intensive growth in the use of medical implants. Besides oral and maxillofacial surgeons, orthopedic, cardiac and plastic surgeons are examples of medical specialists treating millions of patients each year by implanting devices [5]. These medical implants make use of special materials, known as biomaterials.

A biomaterial can be defined as a "material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" [6]. The field of biomaterials resulted from a joint of disciplines including the life sciences, medicine, materials science and engineering [7]. The performance of biomaterials can be classified in terms of: the response of the host to the implant, and the behavior of the material in the host [8]. Ideally, a biomaterial should have specific properties in order to long-term usage in the body without rejection and also have biomechanical properties comparable to those of the autogenous tissues without any adverse effects [6]. In addition to select the right biomaterials for a specific biomedical application some crucial properties are required such corrosion resistance, low toxicity, elastic modulus, fatigue resistance, strength, biocompatibility, bioadhesion, biofunctionality, processability and availability [9] [10]. As a class of materials, metals are widely used in maxillofacial surgery and as dental materials [11]. In the search for an ideal implant material for maxillofacial applications, maximum research has been directed towards titanium in the current century due its biocompatibility and excellent corrosion resistance [12]. However, some metals properties are not sufficient to improve the bonding between implants and tissues, which may result in the inflammatory responses and implants' rejection by the patient. A way for minimize these adverse responses is coating the metallic implant with other materials with different properties like polymers [13]. This class of biomaterials possess unique properties like flexibility, resistance to biochemical attack and good biocompatibility [7]. Biodegradable polymers, like Polycaprolactone, utmost lot of

interest since these biomaterials enabling the surrounding and/or ingrowth tissue to autonomously restore its function and are able to be degraded and excreted or resorbed without the implant removal or surgical revision [14] [15].

1.1 Aim

The main goal of this study is the development of a surface to enhance cell adhesion and the regeneration of connective tissue for maxillofacial damages. Thus this surface will ensure the stability needed of the device for an effective healing process. The biomaterial is composed by titanium grade 2 discs due to its excellent biocompatibility and low corrosion level and by polycaprolactone, a semi-crystalline polymer with slow degradation rate. The Ti discs simulated titanium customized devices that ensure the biomechanical properties necessary to masticatory function and bone stability. The polymeric film should be able to enhance cellular adhesion of the tissue and to avoid the metal surface exposition and corrosion.

The two main objectives for this work are:

- To study film deposition method on titanium discs and subsequently characterize them.
- To characterize the cell adhesion and proliferation to these devices.

Chapter 2 – State of art

2.1 Titanium

2.1.1 Source, manufacturing and application

The first titanium mineral, a black sand called menachanite, was discovered by William Gregor in 1791. Several years later, the element was rediscovered in the ore rutile by a German chemist, Martin Heinrich Klaproth [16].

Titanium is the ninth most abundant element in the earth's crust. It occurs in the minerals ilmenite, rutile and sphene and is present in titanates and many iron ores [17]. The largest use of titanium is in the form of titanium (IV) oxide. It is produced commercially by either the 'sulfate process' or the 'chloride process', both of which use the mineral ilmenite as a starting material. Titanium is produced commercially by reducing titanium(IV) chloride with magnesium [18]. Titanium and its alloys have been used in a wide variety of industrial and commercial applications in such fields as aerospace, architecture, sporting equipment, military hardware, watch manufacture, eyewear, medical implants, and dental products. Commercially pure titanium (Cp Ti) and extra low interstitial Ti–6Al–4V have been mainly used for implant biomaterials among various titanium alloys to date [19] [20] [21]. Some biomedical applications for commercially pure titanium include pacemaker cases, housings for ventricular assist devices, implantable infusion drug pumps, dental implants, maxillofacial and craniofacial implants and screws and staples for spinal surgery [11].

2.1.2 Properties

2.1.2.1 Chemical

Like aluminium and magnesium metal surfaces, titanium metal and its alloys oxidize immediately upon exposure to air. Titanium readily reacts with oxygen at 1200 °C in air, and

at 610 °C in pure oxygen, forming titanium dioxide [22]. Cp Titanium ranges from grade 1, which has the highest corrosion resistance, formability and lowest strength, to grade 4, which offers the highest strength and moderate formability [21]. Chemical composition of Cp Ti are listed in Table 2.1 [23].

Table 2.1 - Chemical composition of Cp Ti grades [23]

	Grade 1	**Grade 2**	**Grade 3**	**Grade 4**
Nitrogen, max.	0.03	0.03	0.05	0.05
Carbon, max.	0.08	0.08	0.08	0.08
Hydrogen, max.	0.015	0.015	0.015	0.015
Iron, max.	0.20	0.30	0.30	0.50
Oxygen, max.	0.18	0.25	0.35	0.40
Titanium	Balance	Balance	Balance	Balance

2.1.2.2 Physical

Titanium is a transition metal light with an atomic number and an atomic weight of 22 and 47.90 g/mol, respectively. Titanium structures have a fatigue limit which guarantees longevity in some applications [24]. Its combination of light weight, strength and its exceptional resistance to corrosion makes it interesting for a great variety of studies in the medical field [25]. Titanium have some important properties that are responsible for its biocompatibility such as low level of electronic conductivity, high corrosion resistance, thermodynamic state at physiological pH values, low ion-formation tendency in aqueous environments, and an isoelectric point of the oxide of 5–6. In addition, the passive-film-covered surface is only slightly negatively charged at physiological pH, and titanium has a dielectric constant comparable to that of water with the consequence that the Coulomb interaction of charged species is similar to that in water [26] [20]. Some important mechanical properties of commercial pure titanium are listed in Table 2.2 [17] [27].

Table 2.2 - Titanium's mechanical properties [28]

	Values
Density (g.cm^{-3}) at 20 °C	4.51
Melting point (°C)	1660
Boiling point (°C)	3287
Phase change point (°C)	882
Thermal conductivity (W/mK)	19.2
Yield strength (MPa)	692
Ultimate tensile strength (MPa)	785
Elastic modulus (GPa)	105
Fatigue life (at room temperature)	19.157

2.1.2.3 Phase

Pure Ti undergoes an allotropic phase transition from hexagonal α-phase (HCP) below 882 ºC to a cubic ß-phase (BCC) over that temperature, as it is shown in Figure 2.1. As its typical microstructure is a single alpha phase, cold work is also an applied strengthening mechanism [16]. These two crystal structures are the basis for naming the three generally accepted classes of titanium alloys: "alpha", "alpha-beta" and "beta" [25].

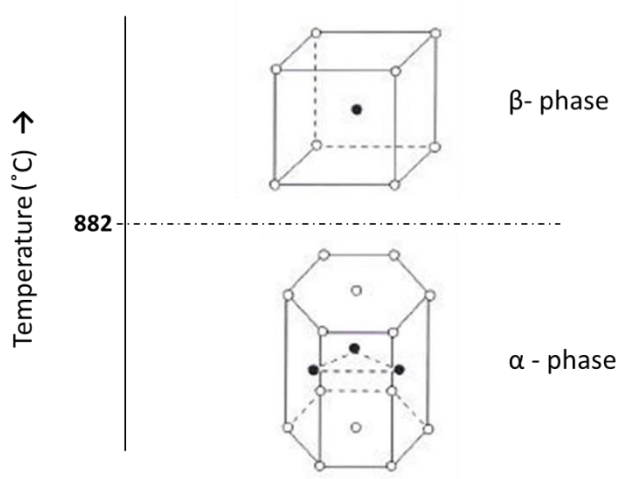

Figure 2.1 - CP titanium crystal structure [25]

2.1.3 Biocompatibility

The events after implantation include interactions between the biological environment and artificial material surfaces, onset of biological reactions, as well as the particular response paths chosen by the body [9].

Commercially pure titanium (Cp Ti) is considered to be the best biocompatible metallic material because its surface properties result in the spontaneous build-up of a 4 stable and inert oxide layer. This oxide layer grows spontaneously in contact with air and it is a powerful barrier against the metal dissolution and is resistant to corrosion [26]. Contrary to some stainless steels, which have induced nickel hypersensitivity in surrounding tissues, titanium does not promote any adverse reactions and is tolerated by the human tissues [21] [25]. The most convincing evidence of the biocompatibility of titanium is the long-term clinical experience with titanium oral implants. Implants success rates within the range of 86-99% after 5-15 years have been reported using a two-stage surgical procedure. As a result of its excellent biocompatibility, titanium interacts well with both hard and soft tissues [25]. However, when subjected to severe mechanical loading, titanium’s oxide film can deteriorate and dissolve,

exposing the unprotected metal to corrosion. Body fluids cannot regenerate the oxide film, and when the unprotected Ti is subjected to corrosion it leaks metal ions and causes clinical problems [29]. Thus, to improve the biological function of Ti implants and the degree of tissue integration, surface modification is employed [9]. Titanium is an interesting material to use in combination with other materials with different properties like coatings such as polymeric films [30] [25].

2.2 Polycaprolactone

Polycaprolactone (PCL) is a polymer with a substantial history of exploration as a degradable material, being easily produced by a variety of routes and capable of surface modification if necessary. Due its flexibility and biodegradability PCL is regarded as a soft and hard tissue compatible material and it has been considered as a potential substrate for wide applications, such as drug delivery, tissue-engineered skin, axonal regeneration and scaffolds for supporting fibroblasts and osteoblasts growth to improve tissue regeneration [31] [32] [33] [34] [35].

Polycaprolactone is linear aliphatic, hydrophobic and semi-crystalline polyester with a high molecular weight that makes it ideal to use it for casting very thin films [36]. This material is prepared by ring-opening polymerization of the cyclic monomer ε-caprolactone (Figure 2.2), and it can be co-polymerized with numerous other monomers because of its high chemical affinity. There are many mechanisms which affect the polymerization of PCL. These mechanisms are anionic, cationic, co-ordination and radical. Each method affects the final molecular weight, molecular weight distribution, end group composition and chemical structure of the polymer [37].

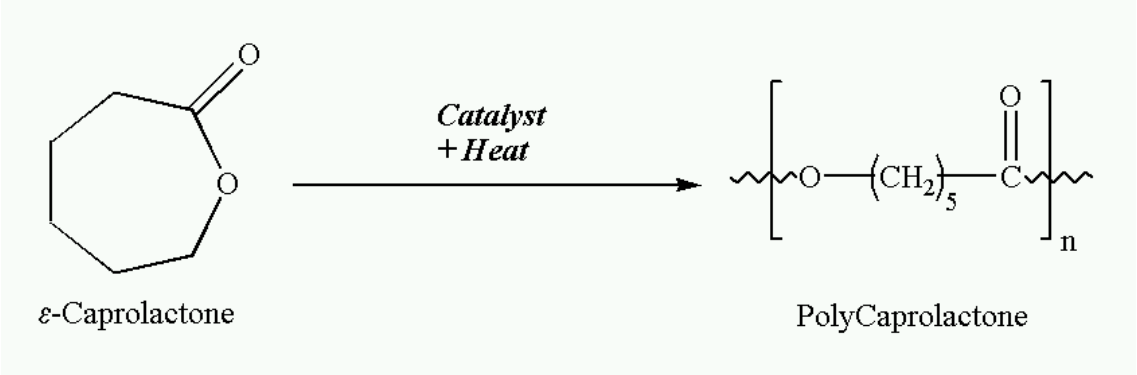

Figure 2.2 - Synthesis of polycaprolactone by ring-opening polymerization (ROP) of ε-caprolactone [37]

Although the main feature of this hydrophobic polyester is its biodegradation. PCL is also remarkable for mechanical properties and easy miscibility with other materials [38]. PCL has low tensile strength (~23 MPa), but high elongation at breakage (4700%) making it a very good

elastomeric biomaterial. Due to PCL's good solubility, *in vivo* low degradation rate, low glass transition temperature (-60 °C), appropriate mechanical properties, low melting point (59–64 °C), high decomposition temperature (350 °C) and easy shaping and manufacture into a large range of implants and devices, it has gained an increased interest in the biomedical field [39] [15]. In addition, a large number of studies show that PCL can be easily combined with other materials like coating metallic implants to further guide the tissue response [40] [41] . PCL is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature. It has a low solubility in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile and is insoluble in alcohol, petroleum ether and diethyl ether [37].

2.2.1 Biodegradation

Polymer degradation was defined by Gopferich in 1996 as the chemical reaction resulting in a cleavage of main chain bonds producing shorter oligomers, monomers, and/or other degradation products [42].

One of its most attractive qualities for PCL use in biomedical applications is its slow degradation rate when compared with other known biodegradable polymers due to its high hydrophobicity and crystallinity [40] [39]. The *in vivo* degradation of poly (ε-caprolactone) was observed for 3 years in rats. The results showed that PCL capsules with molecular weight (Mw) of 66 000 remained intact in shape during 2-year implantation. [43]. PCL degradation occurs in three stages: in the first phase the material goes through a non-enzymatic degradation process resulting in a random hydrolytic cleavage of the ester groups; in the second stage, short-chain oligomers are formed, reducing the PCL molecular weight; in the last stage, the low molecular weight PCL is phagocytosed by macrophages and rapidly degraded in ε-hydroxycaproic acid, which is metabolized into the tricarboxylic acid (TCA) cycle, and excreted by renal filtration [4] [44] [43].

2.2.2 PCL surface modification

Various approaches have been proposed for modifying synthetic hydrophobic biomaterial polymer surfaces [45] [46] [47]. One of these approaches is *alkaline surface treatment* (NaOH) [48]. The benefits of *alkaline surface treatment* on different types of polymers concerning the higher degree of adhesion and proliferation of different cell lines have already been clearly demonstrated [49]. PCL hydrophobicity has hindered its potential for biomedical applications as it hampers cell adhesion to its surface. Several studies have confirmed that the treatment with NaOH improves significantly the hydrophilicity of the PCL films and therefore the interaction with different types of cells [50] [51]. The *alkaline surface treatment*causes the base hydrolysis of PCL esters bonds creating carboxylate ions as illustrated in Figure 2.3. This

creation of functional group will increase the PCL hydrophilicity as well as provide means to covalently attach a bioactive compound in order to improve the cell-implant interaction. NaOH treatment also creates nanoroughness which results in increase of surface area which will enhance cell adhesion [52] [53].

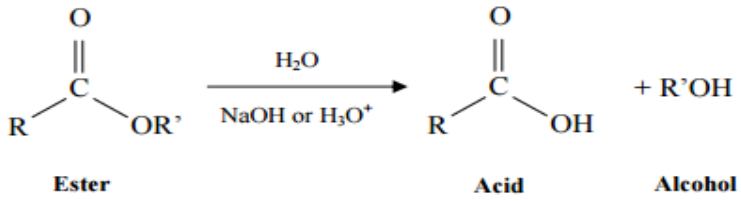

Figure 2.3 - Mechanism of an alkaline hydrolysis of esters [121]

2.2.3 Biomedical application of PCL

PCL was one of the earliest polymers synthesized by the Carothers group in the early 1930s. A resurgence of interest has propelled PCL back into the biomaterials with the birth of a new field, namely tissue engineering [37]. This evolution is depicted graphically in Figure 2.4.

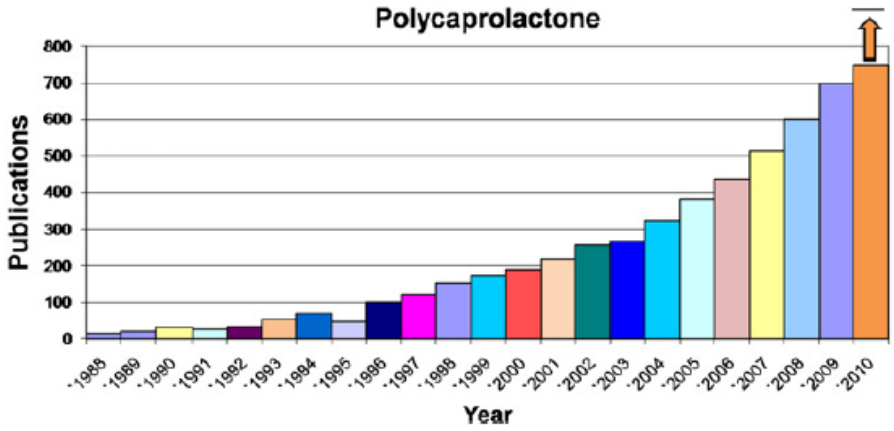

Figure 2.4 - Publications using PCL in the field of Biomaterials or Tissue Engineering during the last 20 years, until April 2010 [37]

PCL is an excellent candidate for copolymerization or blending to engineer desired mechanical properties and degradation kinetics of a medical device or scaffold. A Dutch group were among the first to use PCL-based copolymers to design and commercialize nerve guide devices [37]. Pektok *et al*. studied comparison between ePTFE and PCL grafts, and they proved that small diameter PCL grafts represent a promising alternative for the revascularization procedures because of their better healing properties than ePTFE grafts [54]. PCL is as well suitable for controlled drug delivery due to a high permeability to many drugs and excellent

biocompatibility. Zhang *et al*. studied the potentiality of a new amphiphilic copolymer GC-g-PCL as a potential drug carrier. In this study, DOX was used as a model hydrophobic drug and loaded into this micelle. The authors concluded that the GC-g-PCL micelle was highly efficient at encapsulating DOX, and exhibited a control release profile and effective anti-tumour activity [55]. Moreover, the use of PCL extends to the areas of sutures, wound dressings, contraceptive devices, fixation devices, dentistry and others [37]. A block copolymer of PCL with glycolide, offering reduced stiffness compared with pure polyglycolide, is being sold as a monofilament suture by Ethicon, Inc. (Somerville, NJ), under the trade name Monocryl® [56]. PCL has been also widely used as an ultra-thin film for dressing cutaneous wounds as an homopolymer and also on a combination with other material [57] [58]. In addition, the last two decades have seen substantial research into developing a biodegradable matrix material for controlled release of contraceptives to overcome the necessity of a device replacement surgery [37]. Due to its slow degradation, biocompatibility and FDA approval, PCL is a highly desirable candidate for this role application. Dhanaraju *et al*. have prepared and characterized PCL microspheres, which have inside steroids contraceptive (levonorgestrel - LNG and ethinyl estradiol - EE) to inject intramuscularly into thigh muscle of a rat. The results showed a minimal inflammatory reaction demonstrating that contraceptive steroid-loaded microspheres were biocompatible. This study proved that PCL microspheres can be usedas an injectable implant system for the drug controlled delivery [59]. Lee *et al*. have shown using a poly(L-lactide–co-e-caprolactone) scaffold that if distinct scaffold regions are seeded with appropriate cells harvested from cartilage or ligament sources, complex tissue interfaces like the bone-ligament interface can be regenerated [60]. NaOH-treated PCL films were also investigated by Serrati *et al*. for vascular tissue engineering by supporting the culture of primary vascular cells and endothelial-like EC2 cells derived from endothelial progenitor cells. Results obtained demonstrated that EC2 seeded on NaOH-treated PCL films enhance the basal NO levels and showed a faster and more intense response to physiological stimuli. This could be indicative of a better capacity of EC2 cells to maintain their endothelial functionality when seeded on these polymers [50]. Coupled with relatively inexpensive production routes and FDA approval, PCL represents a promising platform for the design and production of longer-term degradable implants, which could be manipulated physically, chemically and biologically to have tailorable degradation kinetics to suit a specific anatomical site [61].

2.3 Thin Film Production

2D polymeric substrates have been used for *in vitro* cell culture for decades, and surface properties of roughness and topography can be more easily and precisely controlled over a 2D surface than a 3D scaffold [1]. Thin polymer films have attracted much interest in recent years. Thin films offer an alternative solution to increase bioactivity. Their structure can be controlled by melting, crystallization, and wetting [62]. Different processing techniques have been developed for the design and fabrication of thin films. Thin films are formed mostly by deposition, with physical or chemical methods [63]. Among different techniques for organic thin film deposition, spin-coating method has the merit of conveniences, use of low cost equipment and easy and fast materials' production [64].

2.3.1 Spin Coating

Spin coating is a common technique which is widely used for several decades for the application of thin (μm), ultrathin (nm) and homogeneous films onto flat surfaces. This method was first described by Emslie *et al.* (1958) and Meyerhofer *et al.* (1978) [64]. Spin Coating process can be effectively made by dividing it into four stages, as shown in Figure 2.5: deposition, spin up, spin off and film drying. In the first step, a small drop of a solution is deposited onto the center of a substrate with a micropipette. In the second step, the substrate is accelerated to the desired speed. During this stage, the substrate was held firmly in a position by vacuum force. Due to centrifugal force, the solution will spread through the entire surface. In the third step, there is a subsequent reduction in film height because the rotational motion typically results in the expulsion of fluid from the substrate. During final stage, centrifugal outflow stops and further shrinkage is due to solvent loss. After that, a thin film is formed on the surface [65] [66]. One of the most important factors in spin coating is reproduction. Subtle variations in the parameters that define the spin process can result in drastic variations in the coated film. Some important parameters of this technique are spin speed, acceleration and spin time. Spin speed is one of the most important factors in spin coating because the speed of the substrate (rpm) affects the centrifugal force applied to the solution. In particular, the high speed spin step creates a more plane-on orientation and generally defines the final film thickness. The acceleration of the substrate towards the final spin speed can also affect the coated film properties. While the spin process in general provides a radial (outward) force to the solution, it is the acceleration that provides a twisting force into the material. Spin time is a parameter that has to be modulated according to spin speed [64]. Besides the spin coater parameters, there are others that could affect the film thickness as the solution concentration and viscosity [67].

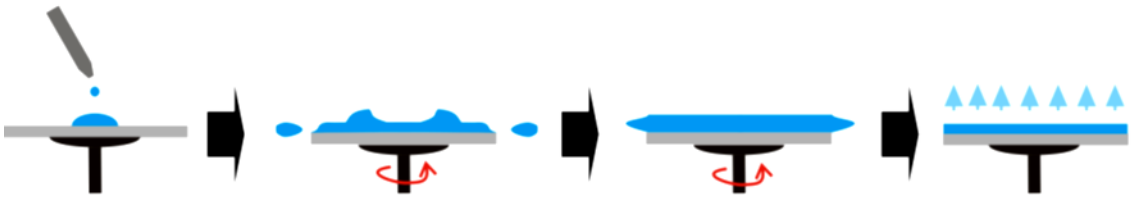

Figure 2.5 - Illustration of spin coating procedure [122]

2.4 Regenerative Medicine and Tissue Engineering: cellular response

Tissues in the human body are the result of millions of years of evolution. This process has resulted in the natural selection of a tissue structure that is optimally adapted to its function in the body [68].

The last two decades have seen a surge in creative ideas and technologies developed to tackle the problem of repairing or replacing diseased and damaged tissues, leading to the emergence of a new field in healthcare technology now referred as tissue engineering [5]. Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [69]. On the other hand, regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function [70].

Tissue remodeling is important for achieving stable biomechanical conditions and vascularization at the host site [71]. In a regeneration strategy, biomaterials promote new tissue formation by providing appropriate surface to enhance and direct cellular attachment, migration, proliferation, desired differentiation on specific cell phenotypes where new tissue formation is needed [72]. Tissue response may be influenced by many factors related to the implant design and localization, physicochemical surface properties, state of the host surgical bed, surgical technique and mechanical loading [73]. Due their similar objectives, the tissue engineering and regenerative medicine fields have been merging in recent years, originating the broad field of tissue engineering and regenerative medicine (TERM) which is focused on the development of alternative therapies for tissue/organ repair [74]. However, within the human body there are tissues with a limited ability of repair/regenerate, posing a challenge that is often difficult for clinicians to overcome. Regeneration of tissues using cells, scaffolds, and growth factors is a key approach in the treatments of injured tissues or organs [71].

2.4.1 Connective Tissue

Connective tissue develops from mesenchyme, a derivative of the mesoderm germ layer [75]. Its function is to maintain the form of the body and its organs and provides cohesion and internal support. This tissue is made up of cells and extracellular matrix. The extracellular matrix is made up of fibers in a protein and polysaccharide matrix, secreted and organized by cells in the extracellular matrix. Variations in the composition of the extracellular matrix, determines the properties of the connective tissue [76]. Regarding to the cells, the type of cells found in connective tissue are fibroblasts, adipocytes, macrophages, mast cells and plasma cells [77].

2.4.2 Fibroblasts

Fibroblasts, one of the most abundant cell type in connective tissues, have different origins at different developmental stages as illustrated in Figure 2.6 [1]. These cells are originally derived from primitive mesenchyme and therefore display the filament protein vimentin, which acts as a marker of mesodermal origin [78]. Fibroblasts play a significant role in epithelial–mesenchymal interactions, secreting various growth factors and cytokines. Its main function is the maintenance of structural integrity of the connective tissue by secreting extracellular matrix and collagen, which form the structural framework of tissues in animals [79]. These cells are large and flat, with elongated processes protruding from the body of each cell, creating the spindle-like appearance of the cell. The nucleus in the body of the cell is oval [80]. In case of injury fibroblasts appear to play an important role in wound healing, and this activity is thought to be regulated by cells known as fibrocytes residing in the tissue stroma. Following tissue injury, they migrate to the site of damage, where they deposit new collagen and facilitate the healing process [78] [81].

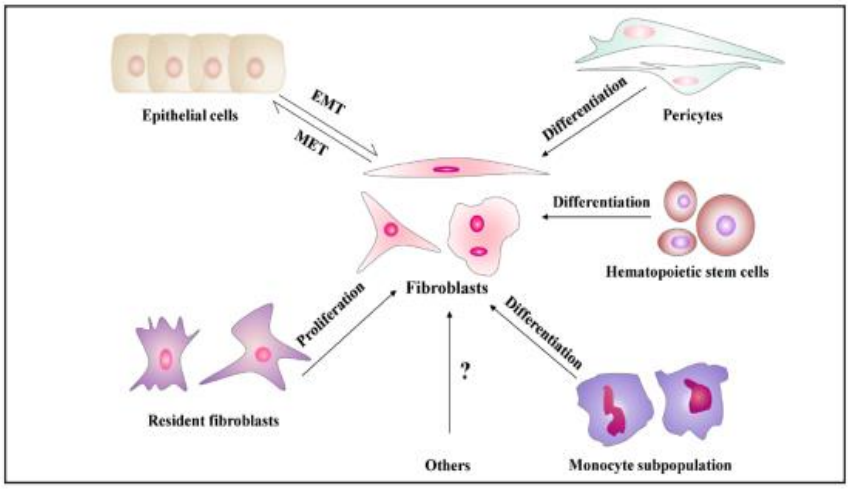

Figure 2.6 - The possible origin of the fibroblasts. The sources of fibroblast include (1) resident fibroblasts division; ; (2) epithelial cells through EMT pathway, fibroblasts in some situations may give rise to epithelia by undergoing MET pathway; (3) hematopoietic stem cell origin; (4) pericytes can contribute to the adult interstitial fibroblast population; (5) monocyte subpopulation can migrate into the damaged parts and differentiate into fibroblasts. [123]

2.4.2.1 L929 mouse fibroblasts and Human Dermal Fibroblasts neonatal (HDFn)

Cell lines are previously established, and generally have origin in transformed primary cultures. Comparatively with primary cultures of the same type of cells, a cell line presents morphological alterations such as decrease cell size, reduced adherence, higher nucleus, and they are most resistant to stress condition than primary cells. These condition allow its use for a long time period representing an abundant source of cell material [75] [82]. L929 is an immortalized mouse fibroblast cell line cloned from strain L.. This cell line is routinely used in

in vitro cytotoxicity assessments due its reproducible growth rate and biological responses [83].

Primary cells on the other hand are not easily available and they do not always exhibit reproducible results due to variation in phenotypic expression from each isolation and loss of their phenotype with the time [84]. These cells are achieved through enzymatic or mechanical disaggregation of a piece of tissue or by spontaneous migration from an explant and may be propagated as an adherent monolayer or as a cell suspension [82]. Dermal fibroblasts are cells within the dermis layer of skin which are responsible for generating connective tissue and allowing the skin to recover from injury. Human dermal fibroblasts neonatal (HDFn) are human dermal fibroblasts isolated from neonatal foreskin. These cells are commonly used in ECM protein analysis, wound healing, collagen metabolism, cosmetics and for skin therapy/models [85].

2.4.3 Wound Healing

A wound is created by any stimulus that disrupts the physical continuity of functional tissues [86]. These disrupts can range from a simple break in the epithelial integrity of the skin or it can be deeper, extending into subcutaneous tissue where other structures such as tendons, muscles, vessels, nerves, parenchymal organs and even bone are damaged [87]. There are two different but simultaneous processes by which wounds can heal: tissue regeneration, the process of returning the site to its original state; tissue repair, the process of generating a scar or less functional tissue of different form and composition than the original. Ideally, the organism response to injury would be regeneration [88].

Wound healing is largely orchestrated by the concerted activity of specialized cells that circulate in the blood in readiness to respond. These cells, collectively called leukocytes, have a characteristic role to play in the resolution of a wound. In addition, other important cells are also involved in healing. Platelets are the first cells to enter the wound and are important mediators of blood clotting [86]. Fibroblasts are the predominant mesenchymal cell type in the cutaneous wound. These cells are responsible to produce the proteins and glycosaminoglycan of the extracellular matrix. The induction of fibroblast proliferation, migration, and protein synthesis is responsible for filling a wound deficit and giving tensile strength to the healed site [89] [86]. Besides the cells action, wound healing is influenced by numerous secreted factors including cytokines, growth factors, and chemokines [90]. The activities of cellular effectors are directed by molecular messengers called cytokines, polypeptides produced and secreted by cells in response to a specific stimulus. These polypeptides are the communications that pass between different types of cells, ordering the strategic performance of specific assignments that coordinate the wound healing [91].

The healing process can be described in terms of four phases: hemostasis, inflammation, proliferation, and maturation/remodeling. For a successful wound healing, all four phases must occur in a proper sequence and time frame [92].

2.4.3.1 Wound healing around implants

In some cases, such as after skin injury, the stages of wound healing can be easily monitored by visual inspection, for example [93]. However, evaluation of the wound healing process around implants can not be accomplished easily or directly.

Biomaterial implantation causes injury to tissues or organs of the host, that will lead to the cascade of inflammation and wound healing, starting with an acute inflammatory response and leading in some cases to a chronic inflammatory response and/or granulation tissue development, foreign-body reaction (FBR), and the formation of a fibrous capsule around the implant [94] [95]. The interface between biomaterial and the body is extremely influenced by the material's surface. Implant surface's properties such as morphology, roughness, chemistry, and geometry (that is, shape and size) strongly influence cellular interactions and tissue integration at the implant site [29]. For example, implant surface topography has a major effect on implant tissue response since it may result in a foreign body reaction, which is characterized by fibrous tissue encapsulation of the implant and the presence of inflammatory cells at the implant–soft tissue interface [40]. Biomaterials adsorb serum-derived opsonins (such as immunoglobulins and activated complement protein fragments) that mediate subsequent adhesion and activation of neutrophils and macrophages. In the attempt to phagocytose the synthetic material, monocytes and macrophages fuse and form multinucleated ''giant cells'' [96]. Besides that, components of the granulation tissue, specifically the new capillaries and fibroblasts, are also part of this response [97] [95]. In addition, the anatomic site of implantation, the adequacy of blood supply at this site, preexisting pathological conditions, and infection are extra factors that affect the wound healing outcome around implants [92]. The magnitude and duration of the inflammatory process has a direct impact on biomaterial stability and compatibility, hence affecting the efficacy of biomedical devices. The ideal outcome is timely resolution of the wound healing process and, most important, integration of the implant into the surrounding biological environment [95].

2.5 Maxillofacial Damages

Every year, hundreds of thousands of people of all ages sustain facial injuries [98]. In recent years, however, traumatic injuries in the elderly have been increasing. This situation occurs due the advances in medicine, resulting in a greater percentage of older people in the population with an active lifestyle [99].

Oral and maxillofacial tissues are a complex array of bone, cartilage, soft tissue, nerves and vasculature. The maxillofacial region can be divided into three parts: the upper face (the frontal bone and frontal sinus), the midface (the nasal, ethmoid, zygomatic and maxillary bones) and the lower face (the mandible) [100] [101]. Damage to these structures, even when minimal, usually leads to noticeable deformities [3]. Maxillofacial fractures include several injuries such as fractures of the lower jaw, upper jaw, palate, cheekbones, nose, and bone surrounding the eyes, skull, or a combination of injuries [98]. These injuries are usually caused by assault, road traffic accidents, falls, sporting accidents and diseases like tumors [101].

Although the principles of treatment are basically the same, substantial differences exist in the response to trauma between the older, the young and middle age populations. The treatment for maxillofacial fractures is influenced by some conditions such as a limited number of the residual teeth, bone atrophy and reduced capacity for tissue repair [99]. This type of trauma may result in life-threatening complications and significant cosmetic or functional problems [102]. During the treatment, some complications may arise such as abnormalities in mastication, swallowing, breathing, smelling, and vision, infection, airway compromise, non-union/malunion of fractures and bone stability loss. The patient may have chronic pain, and those with extensive residual defects frequently develop psychosocial disorders [98][101]. Due these complication, the diagnosis and treatment of these fractures remain a challenge for oral and maxillofacial surgeons, demanding a high level of expertise [99].

Within the past decade, the field of tissue engineering and regenerative medicine has emerged as an exciting alternative for maxillofacial reconstruction [103]. It offers options to supplement existing treatment regimens for reconstruction/regeneration of the oral and craniofacial complex, including the teeth, bones, soft tissues, salivary glands, temporomandibular joint, along with blood vessels, muscles and nerves as well [2].

Chapter 3 – Materials and Methods

3.1 Materials Preparation

3.1.1 Preparation of Titanium discs

Commercially pure Ti (grade 2) discs (granted by INEGI) were cut to an appropriate size (15 mm diameter) as showed in Figure 3.1. The specimens were then cleaned with acetone and ethanol and dried at room temperature overnight for film deposition.

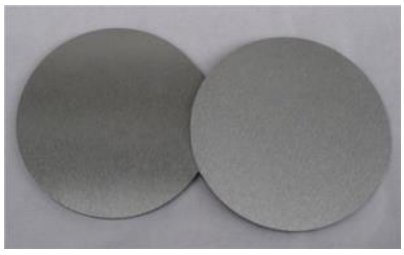

Figure 3.1 - CP Titanium discs grade 2.

3.1.2 PCL solution preparation

The PCL solutions were prepared dissolving polycaprolactone (PCL, Mw = 80,000 from Sigma-Aldrich, Germany) in 20 ml of chloroform (Merck, Germany) in two different concentrations: 7.5% (m/v) and 10% (m/v). These solution were placed in flasks to prevent the solvent evaporation and they were homogenized under magnetic agitation.

3.1.3 PCL film fabrication coating Ti discs

The PCL film coating Ti discs was produced using spin coating technique, previously detailed. Spin coating was carried out in a laminar flow cabinet and 200 µl of each solution were applied on the Ti discs surface with a micropipette. The conditions for spin coating was 3000 rpm for 45s. [70] [72] Excess solution spilled instantly from the substrate surface while spinning, leaving a thin film covering the discs. Subsequently, the samples were allowed to dry on a Petri dish overnight. Two types of Ti discs coated with PCL, with and without NaOH treatment, were produced in order to evaluate if there was significant differences when PCL surface modification was used.

3.1.4 Sodium Hydroxide treatment

In order to improve the PCL surface hydrophilicity, after coated the Ti discs with PCL, some specimens were immersed into a 2 N NaOH (Sigma, Germany) solution at 37◦C for 2 h. After removal from the NaOH solution, the specimens were washed with ultrapure water and dried at 37◦C for a few minutes.

3.2 Surface characterization

3.2.1 Scanning Electron Microscopy

The surface morphology of the Ti discs coated with PCL film, with and without NaOH treatment, was characterized by Scanning Electron Microscopy (SEM). SEM evaluations were carried out with a FEI Quanta 400FEG scanning electron microscope operating at 10KeV. The samples were coated with a thin layer of gold palladium (Bal-Tec-SCD 050).

3.2.2 Atomic Force Microscopy

The surface morphology and mechanical properties of the PCL films, with and without NaOH treatment, was assessed by AFM (Scanning probe microscopy LSPM – AFM/MFM/STM). Topographic images were obtained under Tapping Mode at a scan rate of 1 Hz and 256 scanning lines using a steel cantilever (Veeco DNISP). The samples were analysed using a scan size of 5 µm x 5 µm. For each was performed 16 nanoindentation (4x4). The results were obtained using the NanoScope Software 6.13.

3.3 *In vitro* degradation analysis

The *in vitro* degradation properties were evaluated in Simulated Body Fluid (SBF). This solution was prepared in accordance to Kokubo *et al.*, with ion concentrations nearly equal to those of human blood plasma (Table 3.1) [104]. SBF was buffered at pH 7.4 with tris (hydroxymethyl) aminomethane and 1 M hydrochloric acid (HCl) at 37 °C. Before immersion in SBF solution, the samples were sterilized in ethanol 70% overnight and washed with ultrapure water. Each sample was completely immersed in 17.67 ml of SBF solution. After soaking for 3, 7, 14, 21 and 28 days, samples were removed from SBF, gently washed with ultrapure water and then dried overnight. The samples surface morphology was evaluated by SEM previously described.

Table 3.1 - Ion concentrations of simulated body fluid (SBF) and human blood plasma [104]

Ion Concentrations (m mol)								
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
SBF	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5
Human plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5

3.4 *In vitro* cell studies

To perform the in vitro cell studies the Ti discs coated with PCL, with and without *alkaline surface treatment (2N NaOH)*, were sterilized with ethanol 70% during 30 minutes, washed with PBS twice afterwards and washed with culture medium.

3.4.1 Basal condition of cell culture

Fibroblast L929 cell line (ATCC), subclone of parental strain L, from Mouse C3H/An connective tissue were cultured in α-minimal essential medium (α-MEM) supplemented with 10% v/v fetal bovine serum (FBS, Gibco), 1% penincilin (10µg/ml)/streptomycin (10IU/ml) and 1% fungizone (2,5µg/ml). The cells were seeded on 75 cm^2 culture flasks and then the cultures were incubated in a humidified atmosphere of 5% CO_2 at 37ºC and the medium was changed every 2 days. Human Dermal Fibroblasts neonatal (HDFn) were cultured in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS, Gibco) and 1% penincilin (10µg/ml)/streptomycin (10IU/ml). The cells were seeded on 25 cm^2 culture flasks and then the cultures were incubated in a humidified atmosphere of 5% CO_2 at 37ºC. Fibroblasts L929 and HDFn were maintained until near confluence and the adherent cells were washed with phosphate buffered saline (PBS; Gibco, UK) and enzymatically released with 2 ml and 1 ml of trypsin, respectively, at 37ºC for 5 minutes and counted using a hemocytometer. The resultant

cells were seeded in 24 well culture plates on the Ti discs with PCL films coating, with and without NaOH treatment. The Ti discs without PCL film coating were used as positive control. The fibroblasts L929 were seeded at a density of 1.5×10^4 cells per well in 0.5 ml of α-MEM and the HDFn were seeded at a density of 0.5×10^4 cells per well in 0.5 of DMEM. All cultures were incubated for different time points (1, 3, 7, 10 and 14 days). At each time point, the following tests were performed.

3.4.2 Metabolic Activity assay

The viability and proliferation of the cells on titanium discs (with and without PCL films coating) were determined by the Alamar Blue assay. Fresh medium with 10% of rezasurin was added to the cells and incubated for 3 hours. Afterwards, 100µl were transferred to a 96-well plate and the fluorescence was quantified in a microplate reader (Synergy HT, BioTek) at 530nm excitation wavelength and 590nm emission wavelength. The results were expressed in relative fluorescence units (RFU).

3.4.3 Morphology Analysis

To observe the cells morphology and dispersion under SEM (FEI Quanta 400FEG), the cells were washed with PBS and fixed with 2.5% glutaraldehyde for 30 min. After being washed with PBS to remove the remaining glutaraldehyde, the cells were dehydrated with a graded series of ethanol solutions and hexamethyldisilazane (HMDS, Ted Pella, USA) solutions from 50% to 100%, respectively. The samples were then sputter-coated with palladium-gold.

For cells' morphology evaluation with CLSM (Leica TCP SP2 AOBS) cells were washed and permeabilized with 0.1% v/v Triton X-100 (Sigma, USA) for 30 minutes. After, the cells were washed with 1% BSA for 30 min. The cells F-actin filaments were stained using Alexafluor 488 phalloidin (Invitrogen, USA) for 30 min and the nucleus were stained with a buffer of Propidium iodide and RNase (BD Pharmigen, USA) for 10 min and washed with PBS. The images were acquired with the excitation laser of 488 nm and 594 nm.

3.4.4 DNA Extraction assay

DNA content was measured using the Quant-iTTM Picogreen® DNA assay (Invitogen, UK) according to the manufacturer's instructions. Briefly, at each time point the samples were washed with PBS, frozen at -20º and later thawed at 37º to carry on the measurements. Cells were lized by adding 200µl of 1% v/v Triton X-100 for 30 minutes under agitation at 200 rpm on ice bath. The Quant-iTTM Picogreen® reagent was then added, and the fluorescence intensity was measured with a microplate spectrofluorometer (Synergy HT, BioTek) at 485 nm and 528

nm for excitation and emission, respectively. Total DNA was converted to ρg DNA/sample from a standard curve.

3.4.5 Histochemical

Sirius Red Staining was used to observe the collagen produced by cells in culture (qualitatively). At each time point (7 day and 14 day), the cells were washed with PBS and fixed with 2.5% glutaraldehyde for 15 min. After being washed with PBS to remove the remaining glutaraldehyde, the cells were stained with Sirius Red F3B in saturated aqueous picric acid for 1 hour. Afterward, the Sirius Red stain was removed and the cells were intensively washed with 0.01 N hydrochloric acid (HCl). This reaction stains the collagen fibers in red. The stained samples were analysed in a light microscope (Stereomicroscope (CI 1778)) and an image of each was captured.

3.5 Statistical analysis

The results were expressed as the arithmetic mean ± standard deviation. The statistical analysis of the results was done using the one-way analysis of variance (One-way ANOVA) followed by post hoc Tukey test, with a significance level of $p < 0.05$. The statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc., USA).

Chapter 4 – Results

4.1 Surface characterization

4.1.1 Scanning Electron Microscopy

Samples homogeneity and crystallinity were evaluated by SEM. Figure 4.1 A and B showed SEM images of Ti discs surface coated with PCL before cell seeding. These images showed a Ti surface completely coated by PCL film. The samples presented a homogeneous distribution of the film with the PCL spherulites completely covered the area. It was observed that each spherulite exhibited an extinction cross shape, called a Maltese cross. The samples coated with PCL at 10% (m/v) had a higher spherulites size when compared to samples with lower polymer concentration. These images also revealed that the PCL film surface presented a well-defined semicrystalline structure without any cracks wherein the PCL film at 7.5% (m/v) had less sharp boundaries.

Regarding the samples with *alkaline surface treatment*, Figure 3.1 C and D showed a few cracks on the PCL surface, especially in samples with PCL film concentration at 10% (m/v).

Energy Dispersive X-ray Spectroscopy (EDS) analysis of these samples detected carbon and oxygen from PCL solution and titanium from Ti substrate, as expected (Figure 4.2).

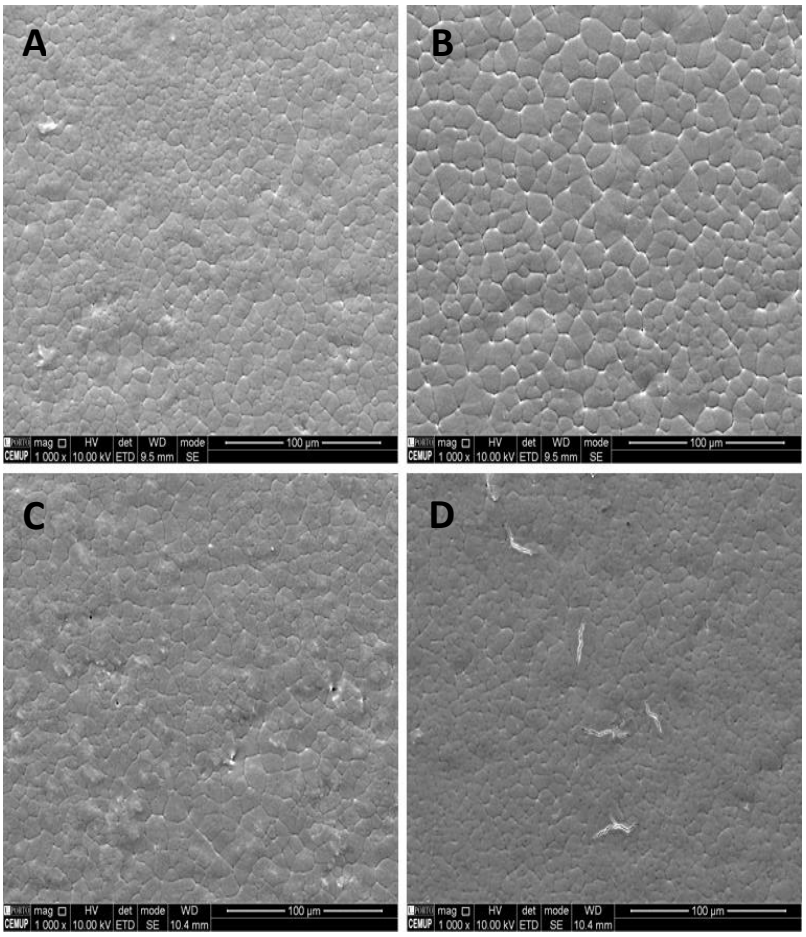

Figure 4.1 - SEM images of the Ti discs surface coated with PCL at (A) 7.5% (m/v), (B) 10% (m/v), (C) 7.5% (m/v) previously immersed in NaOH and (D) 10% (m/v) previously immersed in NaOH. Magnification: x 1000.

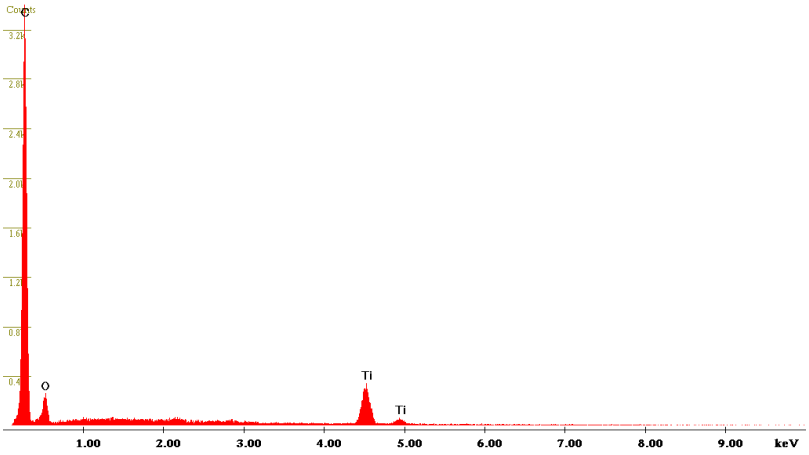

Figure 4.2 - EDS analysis of Ti discs coated with PCL

4.1.2 Atomic Force Microscopy

PCL films' morphology and mechanical properties before and after immersion in NaOH solution were evaluated by AFM. From each sample it was obtained an image to analyze the surface's morphology as illustrated in Figure 4.3. The AFM results showed a homogeneous and smooth surface of samples without NaOH treatment (A and B). The polymer molecules appeared as aggregates of spherulites. The samples with *alkaline surface treatment*, C and D, showed an increase of surface roughness, especially in the samples with lower PCL concentration (7.5% (m/v)).

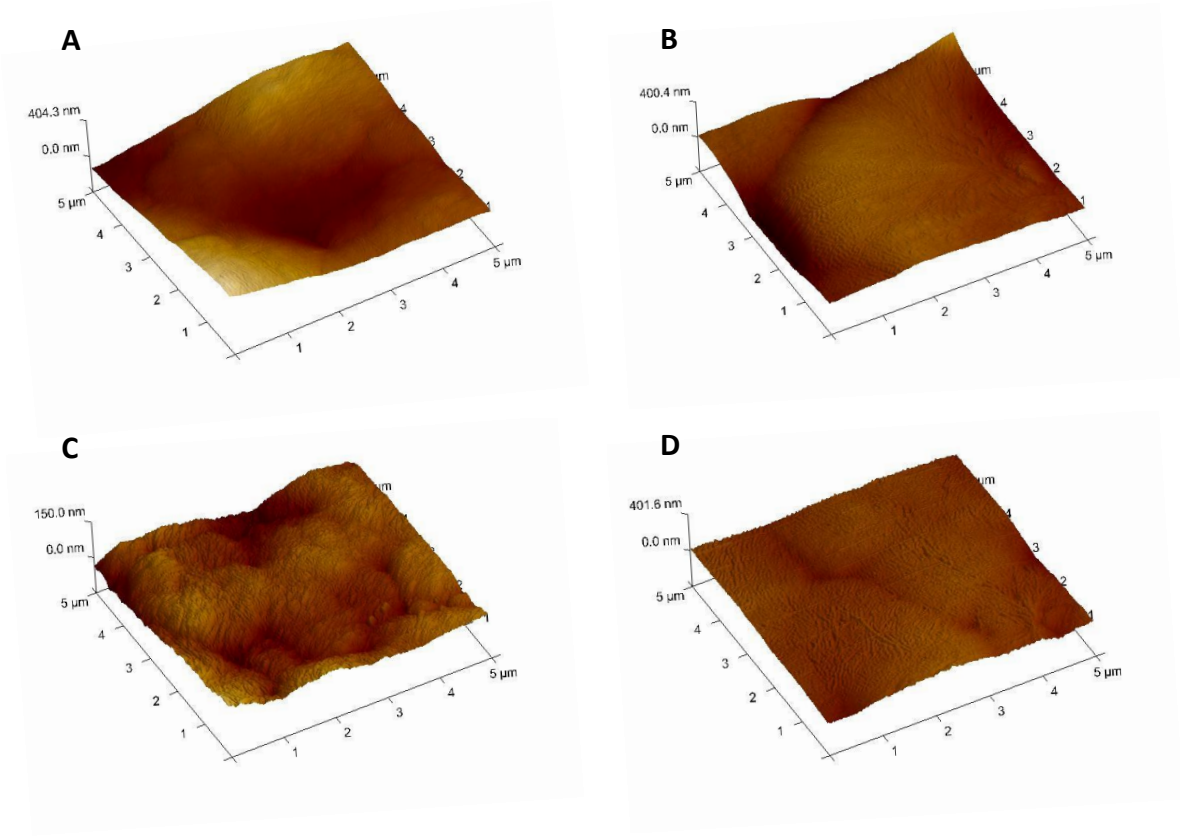

Figure 4.3 - AFM morphology analysis of the Ti discs coated with PCL at (A) 7.5% (m/v), (B) 10% (m/v), (C) 7.5% (m/v) previously immersed in NaOH and (D) 10% (m/v) previously immersed in NaOH.

After nanoindentation process, the young's modulus of each sample was calculated in order to determine if the NaOH treatment influences the mechanical properties of the film. It was noticed that the PCL film's Young's modulus slight decreased after immersion in NaOH solution as showed in Figure 4.4. Specifically, the samples' Young's modulus decreased from 156±17.33 MPa and 155±14.12 MPa for samples coated with PCL at 7.5% and with PCL at 10%, respectively, to 133±9.18 MPa and 134±16.24 MPa for samples with the same PCL concentration and previously treated with NaOH solution. However, this difference is not statistical significant.

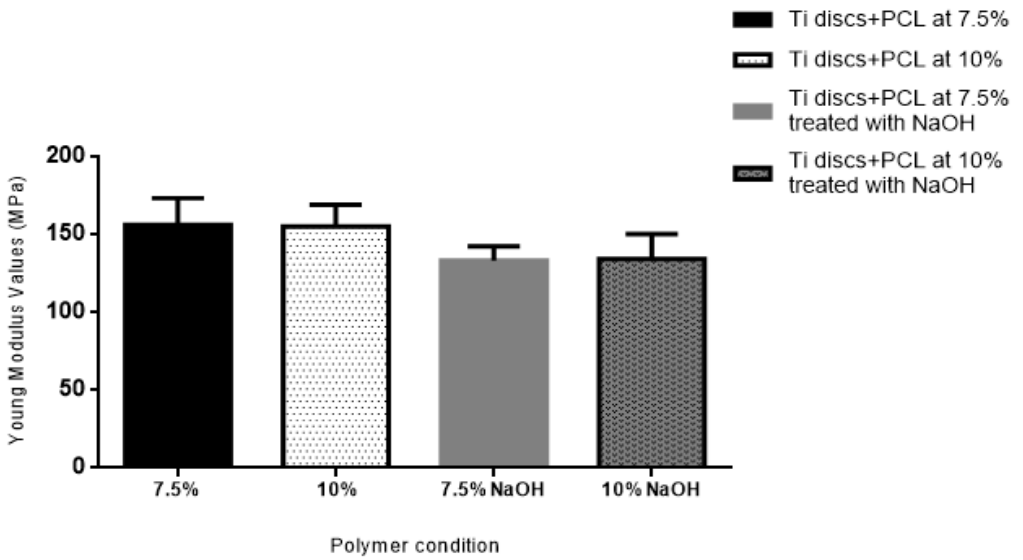

Figure 4.4 - Young's Modulus values of the samples before and after immersion in NaOH

4.2 *In vitro* degradation analysis

4.2.1 Simulated Body Fluid

To evaluate the thin films degradation, Ti discs coated with PCL film were immersed in a SBF for 28 days. In both samples (7.5% and 10% (m/v) at 3000 rpm at 45s) the degradation degree was similar with no severe surface morphology changes as showed in the images of Figure 4.5. However, the degradation degree of the PCL film is more extended in the samples immersed in SBF for longer periods of time. After immersion for 3 days, the samples revealed a slightly eroded surface with loss of some defined limits of crystalline pores. The degradation rate increase until the day 28 where it was visible a higher level of eroded surface and loss of some spherulites sharp boundaries. However, none of the specimens formed apatite-like structures on their surface.

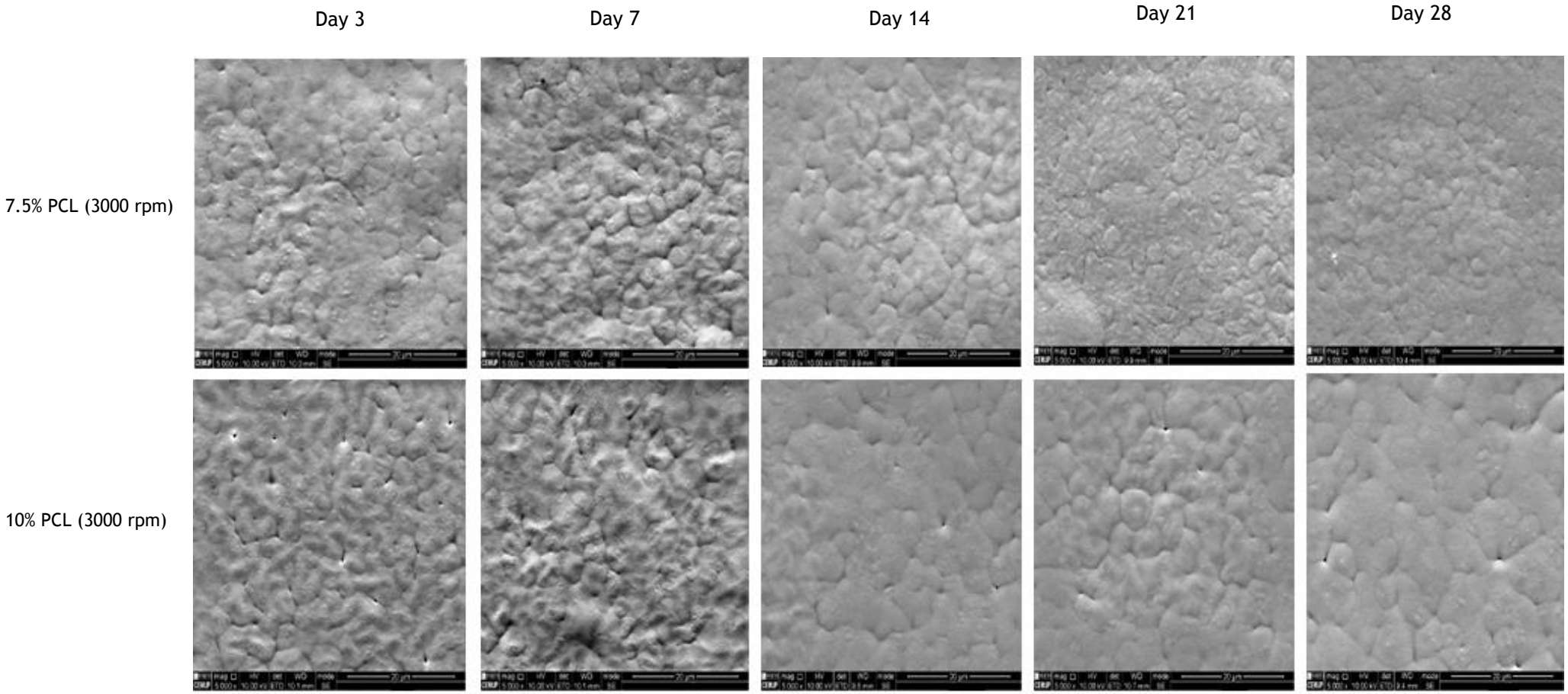

Figure 4.5 - SEM images of the Ti discs surface coated at different polymer concentration after in vitro degradation in SBF for 28 days. Magnification: x 5000.

4.3 *In vitro* cell studies

4.3.1 Metabolic Activity

Figure 4.6 shows the results of the Alamar blue assay for viability and proliferation of Fibroblasts L929. Analysis of cytotoxicity by direct exposure of L929 to the samples showed that the Ti discs coated with PCL, untreated and treated with NaOH solution, had mild or no toxicity. Intra-group analysis showed some significant differences between the samples. Indeed, at days 3 and 7, fibroblasts L929 on the samples with lower PCL concentration (7.5% (m/v)) and *alkaline surface treatment*, exhibited higher proliferation rate with a significant difference ($p<0.05$), when compared to untreated samples at same PCL concentration. Similar results were achieved in the samples coated with PCL at 10% (m/v) on the same day. Inter-group analysis showed an increase in cells viability over time with the cells' metabolic activity maximum reached at day 7. After this day, there was a slight decrease in cells viability in all samples.

Regarding the HDFn cells viability results, Figure 4.7 showed cells' metabolic activity increased with the time of culture. HDFn fibroblasts showed a metabolic activity growth approximately linear with higher cell viability values at longer periods. At day 7, intra-group analysis showed that HDFn cells had higher proliferation on both NaOH treated samples with a significant difference ($p<0.05$) when compared to the untreated samples. However, the cells showed similar cellular viability and still growing in both Ti discs coated with different PCL concentration along the time.

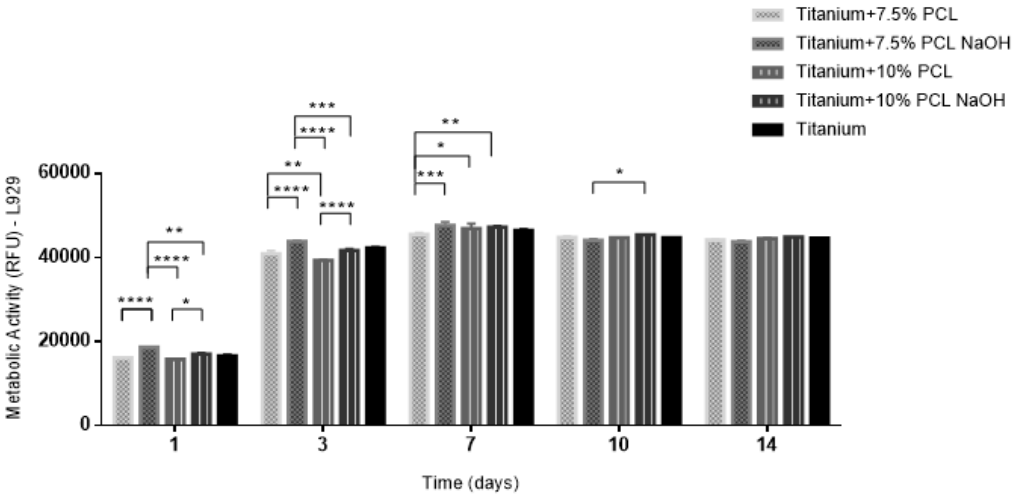

Figure 4.6 - Fibroblasts L929 viability/proliferation at 1, 3, 7, 10 and 14 days using Resazurin. Results presented as average ± SD (n=2). Statistical analysis was performed using a two-way ANOVA. The statistical differences between samples are represented as: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

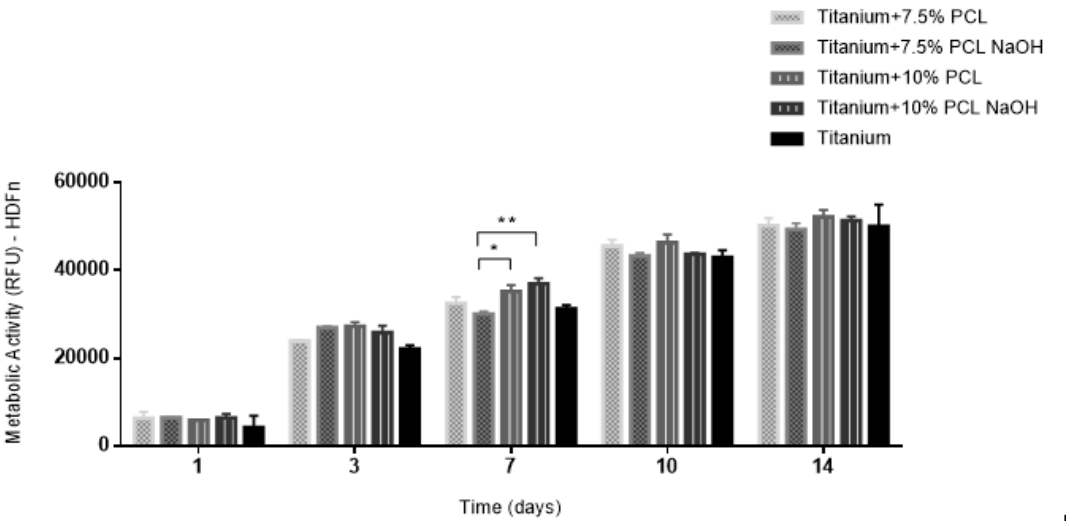

Figure 4.7 - Human Dermal Fibroblasts neonatal viability/proliferation at 1, 3, 7, 10 and 14 days using Resazurin. Results presented as average ± SD (n=2). Statistical analysis was performed using a 2-way ANOVA. The statistical differences between samples are represented as: *p<0.05, **p<0.01

4.3.2 Morphology

To study cells morphology and how the samples support fibroblasts proliferation, L929 and HDFn fibroblasts were cultured on the samples and observed by SEM and CLSM Ti uncoated discs was used as control. SEM observations indicated that fibroblasts L929 cells were well attached, spread and elongated through the thin films surface, while keeping a normal cellular morphology (Figure 4.8). The results also showed that L929 fibroblasts attach and adhere to PCL films slower than to uncoated Ti discs. However, longer culture times show a significant stimulation of cell proliferation on PCL films. In particular, at day 10 the cells reach maximum adhesion and proliferation keeping intact their morphology. It was also noticed that L929 fibroblasts present faster proliferation and more elongated morphology in the samples treated with NaOH solution than in untreated samples, particularly until day 10. Regarding the cells attachment and morphology with the film concentration variation, it was possible to notice that the PCL solution at 10% (m/v) promoted a better and faster adhesion of L929 cells on the film. SEM analysis showed that HDFn cells were well attached and spread through the materials with a spindle-shaped morphology as showed in Figure 4.9. At day 7 and further, the samples' surface were totally covered by the confluent cell monolayer. Thus, there were not significant different in the HDFn cells proliferation in the treated and untreated samples. At day 3, on the contrary, there was an increase in cells proliferation in the NaOH treated Ti discs coated with PCL at 10% (m/v).

On the CLSM observation, a normal cell morphology was identified with the nuclei well distinguished from the cytoplasm, as it can be seen in Figure 4.10 and 4.11. Regarding

fibroblasts L929, at day 1 the majority of the cells showed an oval shape starting the adhesion process. The highest cell proliferation was observed on PCL film at day 7. At this day, fibroblasts L929 has grown into a confluent state and formed a cell to cell connection. It can be seen that cells presented a higher proliferation rate and elongated morphology in the samples with NaOH treatment, more specifically in the Ti discs coating with PCL at 10% (m/v). In the uncoated Ti discs (control group), the cells showed also a high cellular adhesion and proliferation. Regarding CLSM analysis of HDFn cells proliferation and morphology, it was also observed high rate of spread cells on the surface of PCL with a spindle-like morphology increasing along the time (Figure 4.11). The highest cellular proliferation was observed at day 10 with the PCL surface fully covered with a confluent cell monolayer which showed to proliferate in certain regular patterns. From day 7 onwards it was possible to observe that in some areas there is more than one layer of cells which maintain the same orientation, especially in the NaOH treated samples (Figure 4.11, day 14). Overall, HDFn cells proliferation analysis did not reveal significant differences between untreated and treated samples and neither to the uncoated Ti discs (control group).

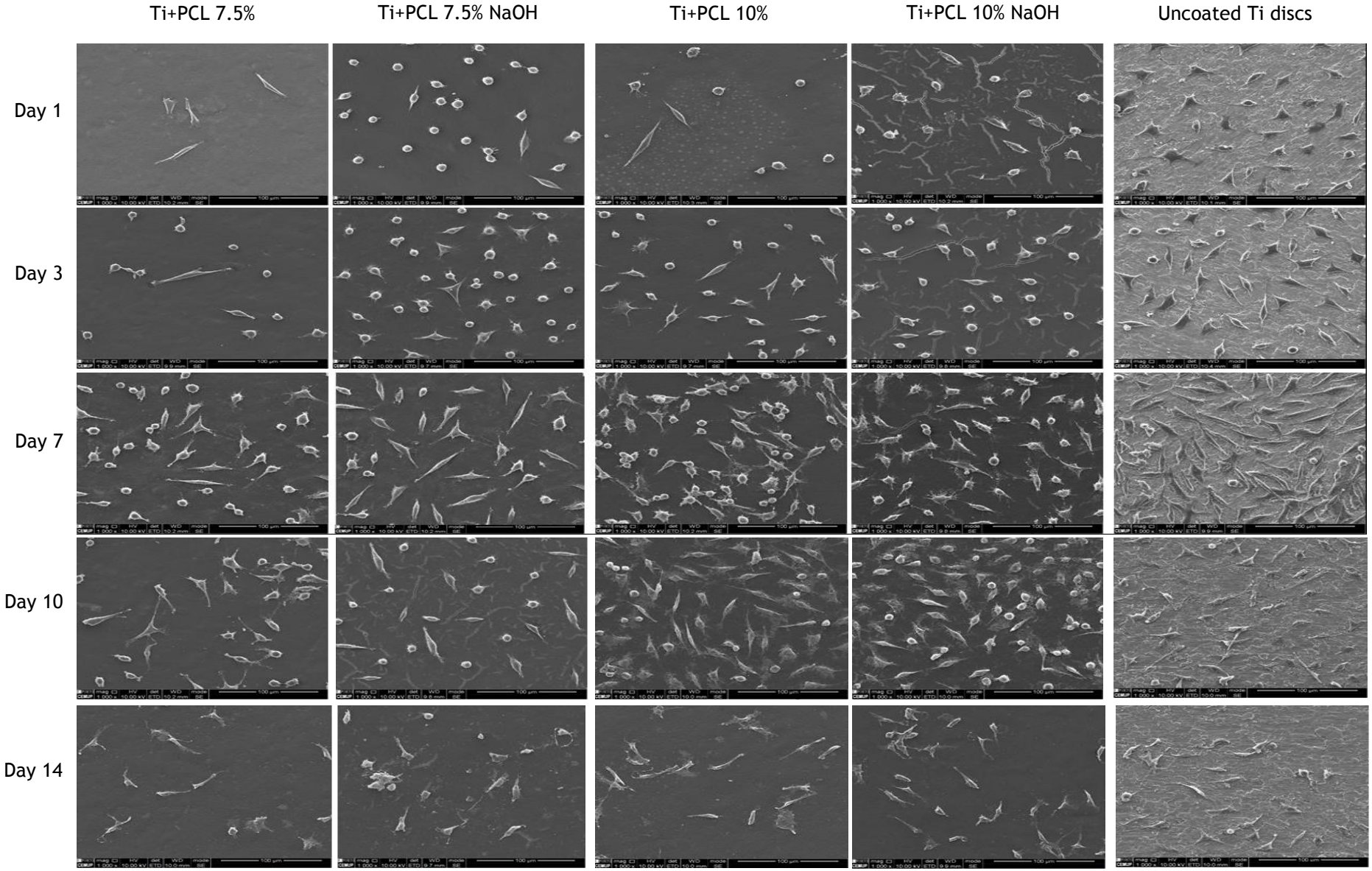

Figure 4.8 - SEM images of Fibroblasts L929 cultured on NaOH treated and untreated Ti discs coated with PCL for 1, 3, 7, 10 and 14 days. Magnification: x 1000.

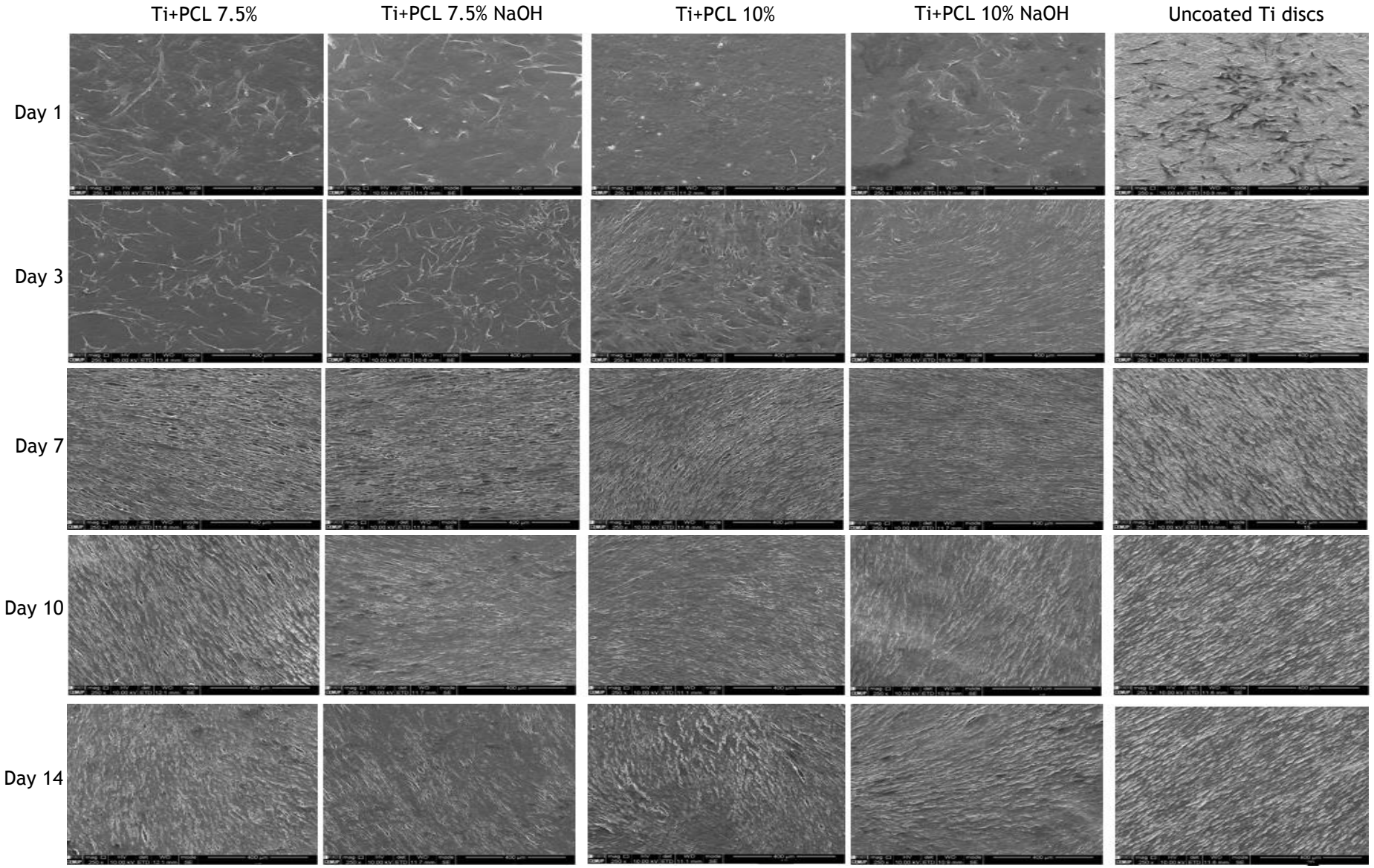

Figure 4.9 - SEM images of HDFn cells cultured on NaOH treated and untreated Ti discs coated with PCL for 1, 3, 7, 10 and 14 days. Magnification: x 250

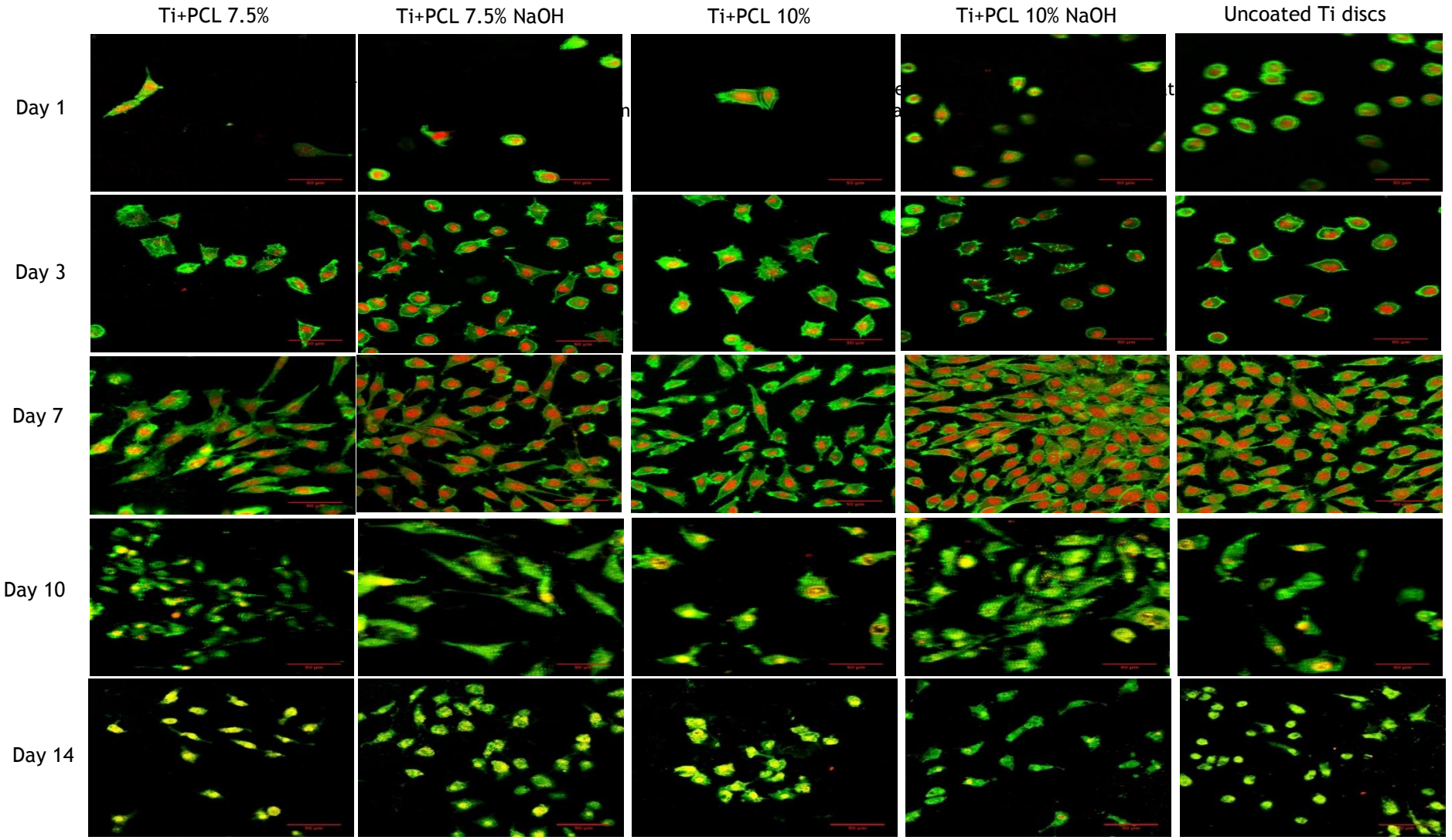

Figure 4.10 - CLSM images of Fibroblasts L929 cultured for 1, 3, 7, 10 and 14 days on NaOH treated and untreated Ti discs coated with PCL. Cytoskeleton is indicated in green while cell nuclei were stained in red.

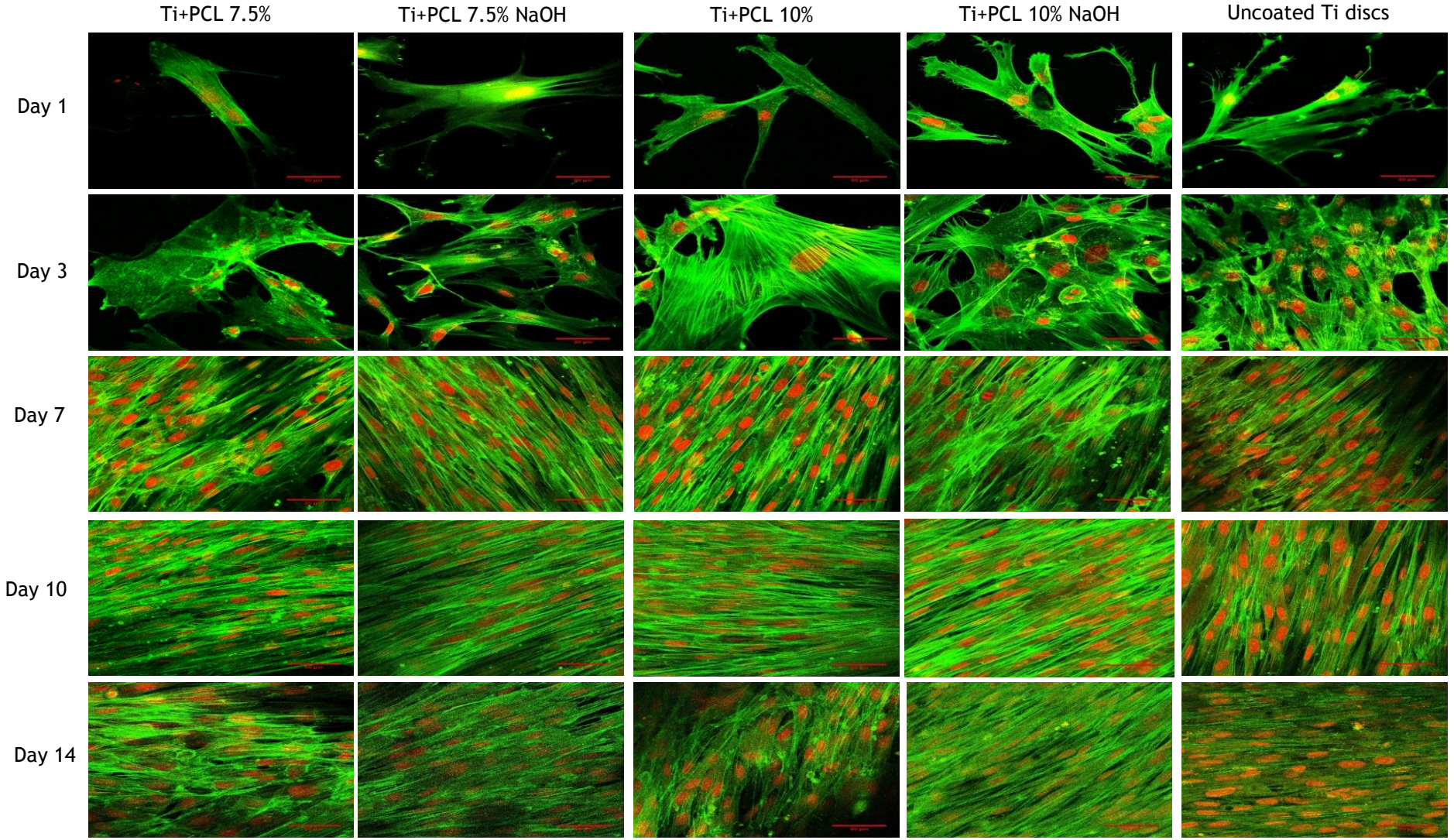

Figure 4.11 - CLSM images of HDFn cells cultured for 1, 3, 7, 10 and 14 days on NaOH treated and untreated Ti discs coated with PCL. Cytoskeleton is indicated in green while cell nuclei were stained in red.

4.3.3 DNA extraction quantification

Cells proliferation was also evaluated by DNA quantification analysis (Figures 4.12 and 4.13). The results regarding fibroblasts L929 demonstrated that there was an increase on the number of cells in all materials until day 7. The enhancement in cell number should give an idea of the proliferation rate of this cell line along the time points. In addition, at this day, the NaOH treated Ti discs coated with PCL film at 7.5% (m/v) revealed the higher cellular concentration. After that, it was noticed a slightly decrease in all samples, same decrease in cell number was observed by SEM and CLSM. HDFn cells had an increase on concentration in all samples over time. It was noticed a higher DNA concentration from day 3. After day 7, the cells proliferation level remained high with no significant difference observed between all the samples.

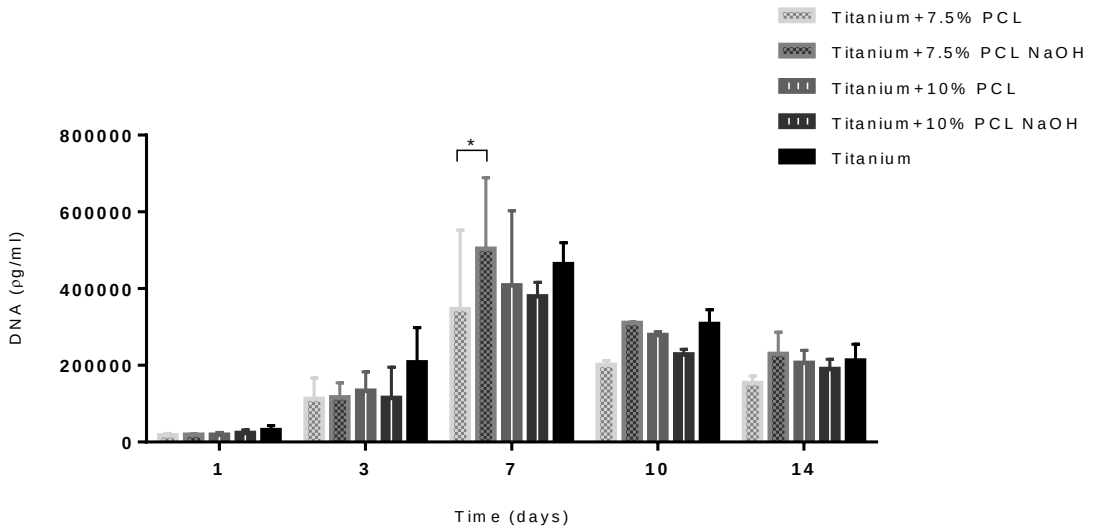

Figure 4.12 - Total DNA extraction quantification of Fibroblasts L929 seeded on Ti discs coated with different concentration of PCL and control (uncoated Ti discs). Differences between the samples were statistically significant ($p<0.05$)

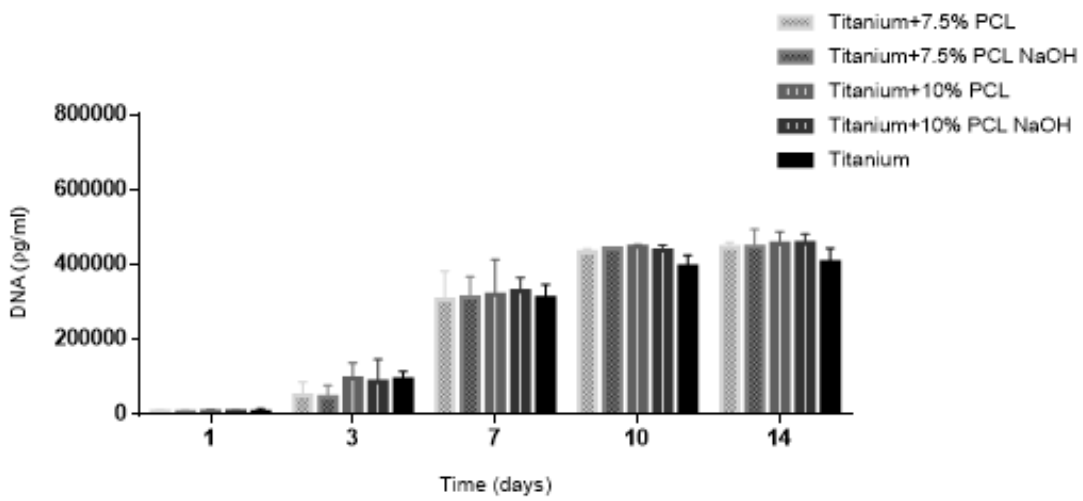

Figure 4.13 - Total DNA extraction quantification of HDFn fibroblasts seeded on Ti discs coated with different concentration of PCL and control (uncoated Ti discs).

4.3.4 Collagen histochemical analysis

Total collagen production quantified by Picrosirius Red staining showed that fibroblasts L929 were able to produce collagen fibers in all materials over time, as can be seen in Figure 4.14. At day 14, it was noticed an increase at collagen amount on the NaOH treated Ti discs coated with PCL film at 10% (m/v) comparatively to other samples.

Regarding HDFn cells collagen organization and production, figure 4.15 showed that fibroblasts were able to produce collagen fibers in both PCL concentration coatings. Indeed, HDFn cells induced a robust deposition of collagen at days 7 and 14. However, at day 14 the fibroblasts revealed a uniform and more expressive distribution of collagen fibers surrounding the cells membranes. For this culture time, the collagen fibers revealed to be oriented in a similar pattern as the cells orientation in the CLMS results (Figure 4.11). The NaOH treated PCL surfaces did not revealed significant effect on the amount of collagen fibers expressed on the different materials.

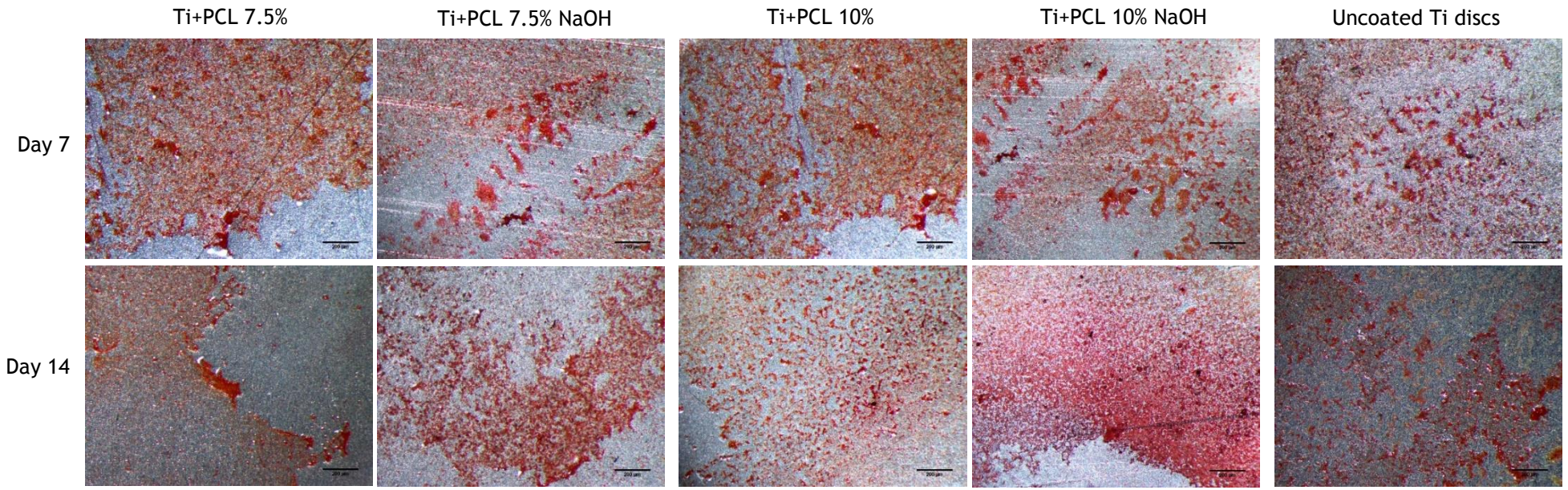

Figure 4.14 - Optical images of Fibroblasts L929 collagen production quantified by Picrosirius Red staining at days 7 and 14.

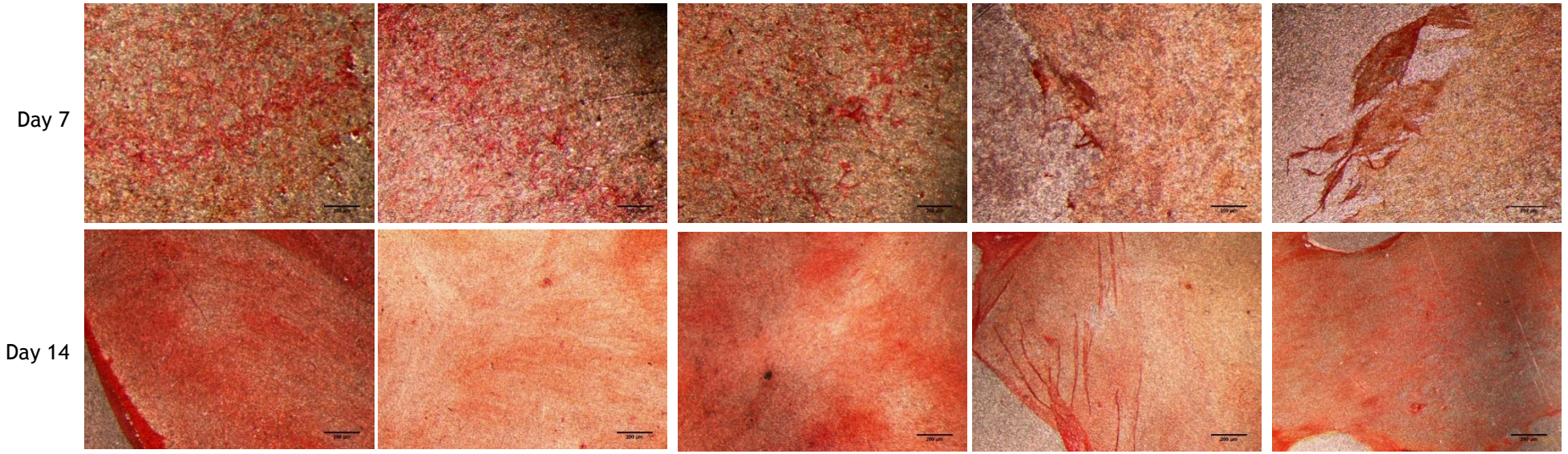

Figure 4.15 - Optical images of HDFn's collagen production quantified by Picrosirius Red staining at days 7 and 14.

Chapter 5 – Discussion

In this study, Titanium Grade 2 discs with 15 mm of diameter were coated with a PCL solution by spin coating technique to restore bone stability and masticatory function and at same time to enhance cell adhesion and promoting new connective tissue regeneration in maxillofacial injuries. For that, it was used two different polymer concentration, 7.5% (m/v) and 10% (m/v) at 3000 rpm at 45s.

PCL is a hydrophobic polymer. This property makes it non-ideal for adsorption of important adhesive proteins (such as fibronectin, vitronectin) in their native conformation. Also PCL's hydrophobic nature could difficult cellular adhesion on the material's surface. Several studies have shown that enhancing the hydrophilic properties of polymers leads to increased cell spreading and adhesion [50] [105]. Thus, to achieve an appropriate behavior (adhesion, proliferation, viability and morphology) of Fibroblasts L929 and Human neonatal Dermal Fibroblasts cultured on PCL films, some specimens were immersed into a 2 N NaOH solution for 2h. The PCL film's alkaline treatment causes ester hydrolysis of the PCL. Suitably this causes formation of –COOH and/or –OH terminated PCL chains. The presence of the hydrolysed ester may be, at least in part, responsible for the observed enhancement of cell adhesion and/or cell proliferation [106]

Surface treatment is a very useful tool in modification of materials' characteristics. This technology is even more important when it comes to implantable biomedical instruments, because specific interactions with *in vivo* environments mostly occur on the surface [107]. In our study, the Ti substrate was well coated with PCL solution creating a thin and homogeneous film that adhered strongly to the substrate, as can be seen in Figure 4.1. The results showed that polymer concentration affect the film morphology once the film at 10% (m/v) demonstrated higher spherulites size with better boundaries shape. This occurs because PCL solution's viscosity increases with the polymer's concentration, controlling the degree of crystallinity of those films that will change the surface uniformity [29]. The surface morphology analysis also revealed the Ti surface completely coated by the spherulites which is characteristic of its semi-crystalline structure. Similar results were achieved by Park *et al*. using magnesium as substrate where after coating with PCL the surfaces showed a number of pores and crystalline structures [108]. After immersion in NaOH solution, Ti discs coated with PCL revealed rough spherulites surfaces and small cracks. Janvikul *et al*. studied the influence of alkaline treatment and plasma treatment in chondrocytes infiltration on PCL scaffolds where they showed similar results on the PCL rough surface after polymer hydrolysis with 1N NaOH

solution [109]. The increase of PCL surface roughness after immersion in NaOH solution was confirmed with AFM analysis [110].

Regarding AFM analysis, the PCL surface's morphology had aggregates of spherulites exhibiting regularly fluctuating rings that was in accordance with the literature [111] [112]. The PCL film surface's analysis before and after NaOH treatment revealed that the alkaline treatment affect the polymer surface morphology. Similarly, Arabi *et al*. demonstrated in their study of surface modification of electrospun PCL fibers that NaOH treatment increased surface roughness which promoted an improvement in cell adhesion [110]. This event occurd due to the carboxyl groups produced when NaOH hydrolyzes ester bonds of PCL. Surface topographic features had been shown to influence intracellular cytoskeleton organization and focal adhesion formation, thus regulating cell adhesion on extracellular substrates [106]. Regarding the Young's modulus calculation, similar results were obtained in both thin films and no significant difference was noticed before and after NaOH treatment. Thus, it is possible to conclude that alkaline surface modification do not change the mechanical properties of the PCL film. Sun *et al*. showed that the Young's modulus PCL films alone was approximately 115.48 ± 9.95 MPa [113]. In our study, the PCL film without NaOH treatment showed a similar value. The small difference between these values is the presence, in our study, of a Titanium substrate under the polymer film.

Besides mechanical properties, other important factor when designing temporary or long-term implants for tissue engineering and regenerative medicine is the materials' biodegradability. The degradation rate of implants have to be slowly enough to be able to complete successfully their function in the body without rejection [14] [75]. PCL degrades much slower than other known biodegradable polymers which makes it very suitable for making long-term medical devices. Indeed, Sun *et al.*, who reported the first long-term in vivo degradation, absorption and excretion studies ever done on PCL, noticed that the *in vivo* degradation of PCL was observed for 3 years in rats. The results showed that PCL capsules with initial molecular weight (Mw) of 66 000 remained intact in shape during 2-year implantation. Afterwards the PCL capsules gradually lost strength and broke into pieces and ultimately excreted from the body through urine and feces [43]. In our study, Ti discs coated with PCL film with two different concentration (7.5% and 10% (m/v)) showed a slower and soft degradation degree. In addition, no significant differences on film degradation in both concentration was noticed. This degradation degree was more evident when immersed in SBF for 28 days. Salgado *et al*. also noticed advanced degrees of degradation in PCL particles when immersed in SBF for 30 days [4]. Similar results were observed in Chouzouri *et al*. study where no drastic changes were evident on the PCL surface after immersion in SBF over a 4 month period [114]. Nevertheless, in our study, it was not observed deposition of apatite-like structures on PCL film during 28 days. Same results were obtained by Yang *et al*. after soaking PCL membranes in SBF during 4 weeks [115]. The same authors also revealed that there was no CaP precipitation visible on PCL membrane in SBF solutions up to 4 weeks of incubation. However, for the nano-apatite/PCL composite, tiny needle-like CaP depositions could be seen on the fiber surface. The incorporation of nAp and its subsequent dissolution, releases calcium ions promoting apatite formation. In this study, no calcification was detected in the PCL film surface, indicating that the system does not promote deposition of apatite. Apatite formation does not occur spontaneously on most synthetic polymers without pretreatment to activate their surfaces [116]. In this study the film was developed for maxillofacial wound repair and did not developed osteoconductivity and bone repair. For these cases, different strategies are available in order

to promote apatite deposition such as the inclusion of bioactive inorganic particles in the material.

Since PCL application in biomedical field is already well detailed, several research work have been developed in order to study the *in vitro* biocompatibility of this polymer using Fibroblasts L929 and HDFn [34] [51] [111] [117]. Fibroblasts L929 are recommended as reference cell line for the cytotoxicity testing of biopolymers. Attachment, adhesion and spreading occur in the first phase of cell/material interactions and will influence the cell capacity to proliferate and to differentiate itself on contact with the biomaterial surface [34]. Different cellular aspects were analyzed in order to observe the cells viability during the culture of fibroblasts L929 and HDFn on PCL films coating Ti discs: adhesion, proliferation, morphology and collagen fibers observation were analyzed. The *in vitro* cell cultures showed that all the thin films were citocompatible and capable of maintaining viable both cells, as it could be seen in Figures 4.6 and 4.7. The comparison of the fibroblast L929 and HDFn's behavior on NaOH treated and untreated materials showed that both cells react differently according to surface roughness and hydrophilicity. However, the fibroblasts L929 demonstrated preference for NaOH treated samples during almost all time points. Some authors suggested that the resulting nanometer surface roughness, due to NaOH treatment, would be the main responsible of this added feature [49] [118]. Overall, the cells revealed high viability and adhesion results when in direct contact with the materials. The quality of cells adhesion will influence their morphology and their capacity for proliferation and differentiation. Fibroblasts cytoskeleton plays an important role in cell motility and shape. The effects of Ti discs coated with PCL on the typical morphology and proliferation of these cells type were carried out by CLSM and SEM studies. At early culture times, both cells cultured on the materials were well adherent and spread out. For long incubation time, it was observed normal cell proliferation with dense multilayers of HDFn fibroblasts while L929 cells reached its maximum elongation in culture faster than HDFn cells, as showed in Figures 4.8 and 4.9. The NaOH treatment effect showed higher influence at early culture time points in both fibroblasts. Altankov *et al*. also demonstrated that more hydrophobic materials significantly reduced endogenous fibronectin deposition, which could lower cell attachment efficacy [119]. At day 3, both NaOH treated samples showed higher density of cells attached on the materials' surfaces, mainly on the PCL at 10% (m/v). The difference between treated and untreated samples was not so significant for long culture times due to cellular confluence. At day 7, both cells were able to proliferate in all samples. Fibroblasts L929 showed cell to cell connection while HDFn cells demonstrated higher proliferation rate over a larger area with a dense multilayered cells where those were observed to align themselves in a specific direction. Ng *et al*. study of PCL thin film for tissue-engineered skin also noticed that HDFn cells proliferate in a certain regular pattern when in contact with different substrates [111]. Regarding fibroblasts L929 proliferation assays, these results were consistent with Serrano *et al*. study where their work provided evidences of good adhesion, growth, viability, morphology and mitochondrial activity of cells on PCL membranes which suggested that PCL is a suitable and biocompatible material for several biomedical application including as a scaffold for vascular graft development [34]. All types of fibroblast are spindle-shaped cells but size difference was obviously observed. Human dermal fibroblasts are much larger than L929 mouse fibroblasts, as can be seen in CLSM results (Figure 4.10 and 4.11). CLSM results also showed high fibroblasts L929 proliferation rate keeping their normal morphology at day 7 and a multilayer of oriented HDFn cells in a regular pattern from day 7. The proliferation results were consistent with those obtained by DNA quantification assay which showed

significant higher fibroblasts L929 concentration at day 7 on the NaOH treated samples while HDFn cells demonstrated higher concentration at day 10 for all samples (Figures 4.12 and 4.13). Collagen type I is the primary constituent of the extra cellular matrix (ECM) of fibroblast cells. Assessing organization of collagen fibers in the ECM of tissues is therefore crucial in developing tissue engineering strategies and the development of biomaterials [120]. In this study, both cells were able to produce collagen fibers in all materials over time. Overall and excluding the NaOH treated sample with PCL at 10% (m/v) at day 14, fibroblasts L929 revealed more intensive collagen fibers deposition at day 7. In addition, these cells showed higher collagen concentration in the samples with higher PCL concentration (10% (m/v)). HDFn cells induced a robust deposition of the fibers due the cells multilayer conformation. Regarding these cells, it was not observed a significant difference on collagen production between the samples which suggested that all materials at all condition were able to ensure collagen fibers production. Comparatively to HDFn cells, fibroblast L929 showed a more heterogeneous collagen fibers distribution.

In the present study, all samples demonstrated good morphological, mechanical and biological properties. However, there was not a material that stands out during all assays. It is important to notice that choosing the best composite for maxillofacial damages depends on the intended application.

Chapter 6 – Conclusion and Perspectives

The main purpose of this present work has been the development of a device composed by titanium discs coated with polycaprolactone at two different concentration. This device should enhance cell adhesion and regenerate new connective tissue, the main goals to achieve after a maxillofacial injury. When observed by SEM, the obtained biomaterial presented a homogeneous surface in both PCL concentrations with no drastic changes in its morphology after immersion in SBF for 28 days. The AFM analysis showed no significant difference in Young's modulus value between samples before and after NaOH immersion (similar mechanical behavior). In the different cell cultures, it was confirmed that the Ti coating produced was biocompatible and allowed cell adhesion, viability and proliferation. Both NaOH treated and untreated PCL film's concentration allowed high cell proliferation and were capable to keep the fibroblasts morphological shape. Comparatively to HDFn cells, fibroblasts L929 demonstrated better proliferation results in NaOH treated samples due the increase of surface hydrophilicity. In addition, SEM and CLSM results showed that fibroblasts L929 demonstrated higher proliferation rate and morphological results in the samples with higher PCL concentration (10% (m/v)). HDFn fibroblasts showed higher proliferation rate and attachment results along the days. Collagen organization analysis showed a robust production of collagen fibers, mainly in HDFn cells, increasing along the time of culture. Once again, there was no significant differences in total collagen amount on NaOH treated and untreated samples. These results showed that the Ti discs coated with PCL film at 7.5% and 10% (m/v) provided adequate environment for cell adhesion and proliferation, improving cell response. The combination of these cell culture results, in addition with normal morphology and mechanical properties results, leads to the conclusion that the this surface treatment is potentially novel candidate that can be use in maxillofacial field in order to improve and enhance cell adhesion to promote good tissue regeneration.

Taking into account the results obtained during this work there are some aspects and ideas that can be explored and implemented as future work that could improve the performance of the desired device. Such are:

- Pretreated titanium discs using alkaline or acid attack before coating with PCL so that after polymer layer degradation the metal surface could be protected from corrosion.

- Although *in vitro* test has given some preliminary guide lines about cell biocompatibility, it is still necessary to obtain a much clear idea about the host tissue response to the composite after *in vivo* implantation.

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