Universidade do Minho
Escola de Engenharia

David Losa de Areia Afonso

Fish Scales as Model for Osteogenic Cells Culture

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Dissertação de Mestrado
Ciclo de Estudos Integrados Conducentes ao Grau de Mestre em Engenharia Biomédica
Área de Especialização em Biomateriais, Reabilitação e Biomecânica

Trabalho realizado sob a orientação do
Professor Doutor Rui Luís Reis

Outubro de 2011

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Título dissertação: Fish Scales as Model for Osteogenic Cells Culture

Ano de conclusão: 2011

Orientador: Professor Doutor Rui L. Reis

Designação do Mestrado: Ciclo de Estudos Integrados Conducentes ao Grau de Mestre em Engenharia Biomédica

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Escola de Engenharia

Departamento de Polímeros

Guimarães, _____/_____/_____

Assinatura: _____

Acknowledgements

I would like to acknowledge to all that somehow have contributed for the development of my work along this year and welfare, especially to:

- My parents, José Afonso and Margarida Afonso, for their support and advisement during my whole life. Also my brothers, other members of my family (including Elisa), as well as my friends that were crucial in my growth as a person.

- Professor Rui L. Reis for the opportunity of developing my work in a place of excellence such as 3B's.

- Professor Antonella Motta, who was my tutor and pathfinder, guiding me in order to achieve our objectives for this Project.

- Dra. Joana Moreira-Silva, Dr Tiago H. Silva and Dra. Alexandra P. Marques for their help, availability, support and guidance during the time i worked in 3B's.

- Professor Claudio Migliaresi for receiving and supporting me during my ERASMUS period in a laboratory with extremely good conditions to work.

- Professor João Mano for his advisement and for giving me the opportunity to study abroad.

- Dr. Fábio Zomer Volpato, who was many times my teacher, helping me to learn how to work and know my way around a laboratory.

- My colleagues / friends of Biomedical Engineering for the best 5 years of my life where we shared so many stories that I will never forget.

- All my ERASMUS friends for their friendship and entertaining times we spent together, especially Nuno, who was my partner along my Erasmus journey.

- All the researchers and workers both from the 3B´s and BIOtech, who were always ready to help me in all kinds of situations, especially: Lara Reys and Rogério Pirraco.

Abstract

Considering the fish scales composition of hydroxyapatite and type I collagen fibrils, the organized and regular pattern of the scales, and also their capability to provide a form of armor plating to protect fish from injury and disease transmission, its potential biomedical application might be striking. In this work, fish scales of the specie *Lates Calcarifer*, also known as White Seabass, were studied, in particular by the assessment of their potential as model for osteogenic cells culture.

For this purpose the fish scales were collected in a market in Thailand. The scales characterization, especially concerning their pattern, was done by observations in SEM and Confocal Microscope. Results showed that the overall scales pattern is formed by parallel concentric lines and is highly composed of collagen. An *in vitro* biological analysis of the scales when used as films to support cell (osteoblasts) proliferation was also done. In order to infer about the scales patterning potential on the proliferation of cells, fibroin films with the same pattern of the fish scales were produced, as well as and films without any pattern. To assess cell viability and cell proliferation, a technique denominated Alamar Blue was performed, being the cell proliferation higher on the scales, comparing with the fibroin films. Cell adhesion morphology, cytoskeleton architecture and alignment were evaluated by observations in the SEM and Confocal Microscope. The osteoblasts presented an orientation according to the fish scales pattern alignment and acquired an elongated and narrow shape. The DNA quantification and ALP test allowed drawing conclusions about the cell activity. The cells on the fibroin samples did not show any ALP activity during the 14 days of proliferation, although the ones on the scales already showed some activity after 14 days of the cells seeding.

An *in vitro* biological analysis of the fish scales using human adipose-derived stem cells (hASCs) was executed, in the presence or absence of osteogenic differentiation factors. The stem cells behavior was very similar to the osteoblasts, presenting a strait and elongated shape aligned with the pattern axis.

Results suggest that the fish scales composition and topography are responsible for a good cell proliferation and induce modifications in cell organization and morphology.

Resumo

Tendo em conta a composição das escamas de peixe em hidroxiapatite e fibras de colagénio do tipo I, o padrão organizado e regular das escamas, e também a sua capacidade de proporcionar uma forma de blindagem para proteger o peixe de lesões e transmissão de doenças, a sua aplicação biomédica poderá ser espantosa. Neste trabalho foram estudadas escamas de peixe da espécie *Lates Calcarifer*, referente ao Robalo Asiático, em particular a avaliação do seu potencial como modelos para cultura de células osteogénicas.

Para este efeito, as escamas de peixe foram trazidas de um mercado na Tailândia. A caracterização das escamas, tendo predominantemente em conta o seu padrão, foi feita através de observações no SEM e Microscópio Confocal. Os resultados mostraram que o padrão global das escamas é constituído por linhas concêntricas paralelas e altamente composto por colagénio. Uma análise biológica *in vitro* das escamas quando usadas como *scaffolds* para suporte de proliferação celular (osteoblastos) também foi realizada. A fim de inferir acerca do potencial das escamas na proliferação dessas células foram também produzidos filmes de fibroína com o mesmo padrão das escamas e filmes sem qualquer padrão. Para avaliar a viabilidade e proliferação celular foi realizada uma técnica denominada "Alamar Blue", tendo sido a proliferação celular maior nas escamas comparando com os filmes de fibroína. A morfologia de adesão celular, arquitectura do citoesqueleto e alinhamento foram avaliados por observações no SEM e Microscópio Confocal. Os osteoblastos apresentaram uma orientação de acordo com o alinhamento do padrão das escamas e adquiriram uma forma alongada e estreita. A quantificação de DNA e teste de ALP permitiram tirar conclusões acerca da actividade celular. As células nas amostras de fibroína não mostraram qualquer actividade celular durante os 14 dias de proliferação, contudo nas escamas mostraram já alguma actividade 14 dias depois do início da cultura das células.

Uma nova análise *in vitro* das escamas foi feita com células estaminais do tecido adiposo, na presença e ausência de factores de diferenciação osteogénica. O comportamento das células estaminais foi semelhante ao dos osteoblastos, apresentando uma forma estreita e alongada e alinhadas com o eixo do padrão.

Os resultados sugerem que a composição e topografia das escamas são responsáveis por boa proliferação celular e por induzirem modificações na morfologia e organização celular.

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Abbreviations

A

ALP - Alkaline Phosphatase

B

BSE – Bovine Spongiform Encephalopathy

BCA - Bicinchoninic Acid

BMSC – Bone Mesenchymal Stem Cells

D

DSC - Differential Scanning Calorimetry

DNA – Deoxyribonucleic Acid

DAPI - 4'-6-Diamidino-2-phenylindole

E

EDTA - Ethylenediamine tetraacetic acid

ECM – Extracellular Matrix

F

FMD - Foot and Mouth Disease

H

hASCs – Human Adipose derived Stem Cells

HAp - Hidroxyapatite

M

MDSC - Modulate Differential Scanning Calorimetry

MEM – Minimum Essential Medium

P

PEG – Polyethylene Glycol

PDLLA – Polylactic Acid

PBS – Phosphate Buffered Saline

S

SEM – Scanning Electron Microscope

T

TSE - Transmissible Spongiform Encephalopathy

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CHAPTER I.

GENERAL INTRODUCTION

1 | Motivation and outline

"Biomimetics" is a recent definition but in fact the humankind was inspired by Nature since the beginning of civilization. Creatures and plants possess tools and skills that are attractive to mimic. Mimicking nature cannot be a goal in itself, once this would hardly represent progress. The goal is to learn its principles, extract the general physic-chemical processes of the object/principle to be mimicked, and thereafter transfer it to a synthetic process or new material [1]. Fish scales study is relevant in order to extract some principles for appliance in different areas, being the biomedicine a potential *environment* for this praxis considering the fish scales composition, structure and functions.

There are many bone diseases that can affect and condition at many levels people's lives. The traditional existent treatments are not, in many cases, an effective response for these problems, mainly because the bone ability to recover and regenerate is limited. Therefore the search for alternative and more complex methods for the treatment of bone diseases is a field where many developments can be achieved as a response to improve many people's life quality. Bone tissue engineering is a developing field, being an attractive approach with great potential for repairing bone defects since it is based in the body's natural biological response combined with engineering principles.

Tissue engineering is a rich and challenging area to be explored. To achieve great progress many small/big steps still have to be undertaken, and the testing of many hypotheses to be made. The fish scales study fits in this context, with great potential, as it will be mentioned later.

The aims of this project were the characterization of fish scales of *Lates Calcarifer* more concerning their pattern, and the study of the scales as scaffolds with the assessment of the cells behavior such as proliferation, adhesion, alignment and activity. The cells used for this study were both osteoblasts and, in a later phase, human adipose derived stem cells (hASCs). A first study using osteoblasts involved the utilization of 3 groups of fish scales, differing in the treatments they were submitted to, and fibroin films with the same surface pattern found in the scales, following a biomimetic approach, and without any pattern. In the study of the role of the fish scales pattern over hASCs, collagen films with the scales pattern were also prepared, and the cells behavior in the presence and absence of osteogenic differentiation factors were compared.

2 | Bone morphology, problems and therapeutic approaches

Bone can be regarded as a composite material composed by cells, organic and inorganic matrices, and an extracellular matrix (ECM). Bone cells can be further subdivided in osteoblasts (the bone forming cells), osteocytes (the main cells of fully formed bone), and osteoclasts (the multinucleated giant cells responsible for bone resorption). The organic matrix (approximately 35% of the dry weight of bone) is mainly constituted of type I collagen (approximately 90%). Collagen is organized in fibers in which mineralized tissue is deposited [2] [3]. The inorganic matrix (approximately 60%-70% of the dry weight of bone) provides minerals for homeostasis, such as Ca, O, Na and Mg, and it consists of hydroxyapatite (HAp). This family of materials also contains other members, like dentin (material that constitutes the inner layers of teeth) and cementum (thin layer that binds the roots of teeth to the jaw) [4] [5] [6]. The ECM is composed by non-collagenous proteins, which are responsible for providing the linking structure and function of cells with soluble signaling factors, thus affecting cell anchorage, proliferation and expression [7] [8] [9].

Typically, the adult skeleton contains 80% cortical (compact) bone and 20% trabecular bone. Cortical bone is hard and dense and makes up the shaft surrounding the marrow cavity of long bones as well as the outer shell of some other bones. Cortical bone is only 10% porous, allowing room for only a small number of cells and blood vessels. The structural unit of cortical bone is a cylindrically shaped osteon, which is composed of concentric layers of bone called lamellae. Blood vessels run through Haversian canals located at the center of each osteon while nutrient diffusion is further aided by canaliculi, or microscale canals within bone. Osteons are aligned in the longitudinal direction of bone and, therefore, cortical bone is anisotropic [2] [3] [4] [5] [6] [7].

Bone tissue is responsible for assuring functional properties, such as mechanical support and protection of the organs, and a site of attachment for muscles used in locomotion, as well as physiological activities, such as responsiveness to metabolic needs [4] [10]. It also acts as a deposit for phosphate and calcium in the preservation of normal mineral homeostasis [6].

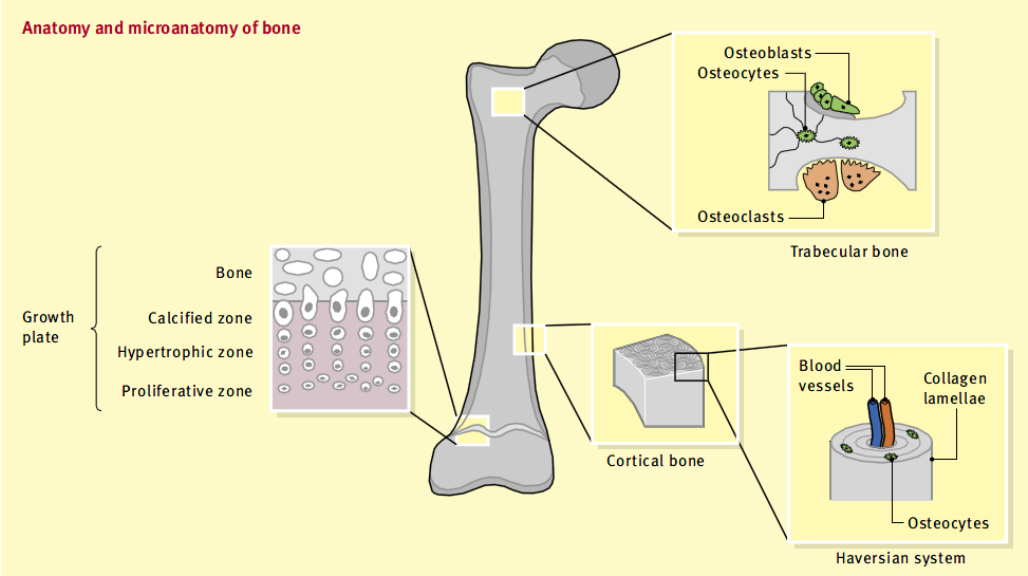

Figure 1,1 - Anatomy and microanatomy of bone (adapted from Ralston, Stuart H. Structure and metabolism of bone. Medicine. 2005)

Bone is continuously remodeled during the lifespan of most vertebrates, including man. Bone remodeling is the result of the balance between the activities of two different cell populations, the osteoclasts and the osteoblasts that are responsible for bone resorption and deposition, respectively. Bone lesions and defects occur in an ample variety of clinical situations, and their reconstruction to provide mechanical and functional integrity is an essential step in the patient's rehabilitation. The potential of bone to regenerate spontaneously many bone lesions, such as fractures, allow the healing with conventional conservative therapy or surgery. Nevertheless, bone diseases are many and quite different. Current available options for treatment of orthopedic diseases include grafts and synthetic materials, with their respective advantages and drawbacks. In the treatment of some bone diseases, biomaterials play an important role and constitute an outstanding improvement in the quality of life of a large amount of patients, who would otherwise be confined to their most basic activities and limited to a painful life. Such diseases include skeletal loss due to trauma or removal of tumors, fractures and joint malfunctioning, as well as a wide spectrum of specific bone diseases [5] [7] [11] [12] .

The deployment of bone tissue engineering has made it an attractive forthcoming with great potential for repairing bone defects resulted from trauma, surgical resection and congenital deformity corrections. Bone tissue engineering provides a promising alternative strategy of healing severe bone injuries by using the body's natural biological response to tissue damage in

conjunction with engineering principles. Osteogenic cells, biomaterial scaffolds, and growth factors are the ground base of the many bone tissue engineering strategies applied to achieve repair and restoration of damaged tissue. An ideal biomaterial scaffold will offer mechanical support in an injured site and simultaneously deliver growth factors and cells into the defect to encourage tissue growth. Besides that, this biomaterial should degrade in a controlled manner without causing a significant inflammatory response in order to restore damaged bone structurally and functionally [7] [10] [13] [14] [15] [16].

3 | Fish Scales: Morphology and Potential

This study focuses on the fish scales of a species denominated *Lates Calcarifer*, which is one of the nine *Lates* species of the family *Centropomidae* [17]. The normal maximum size of these fish is about 120 cm but they can grow up to 180 cm [18].

Like many other structural biological materials, the fish scales structure displays a characteristic hierarchical structure, built over several distinct length scales (Figure 1.2) [19].

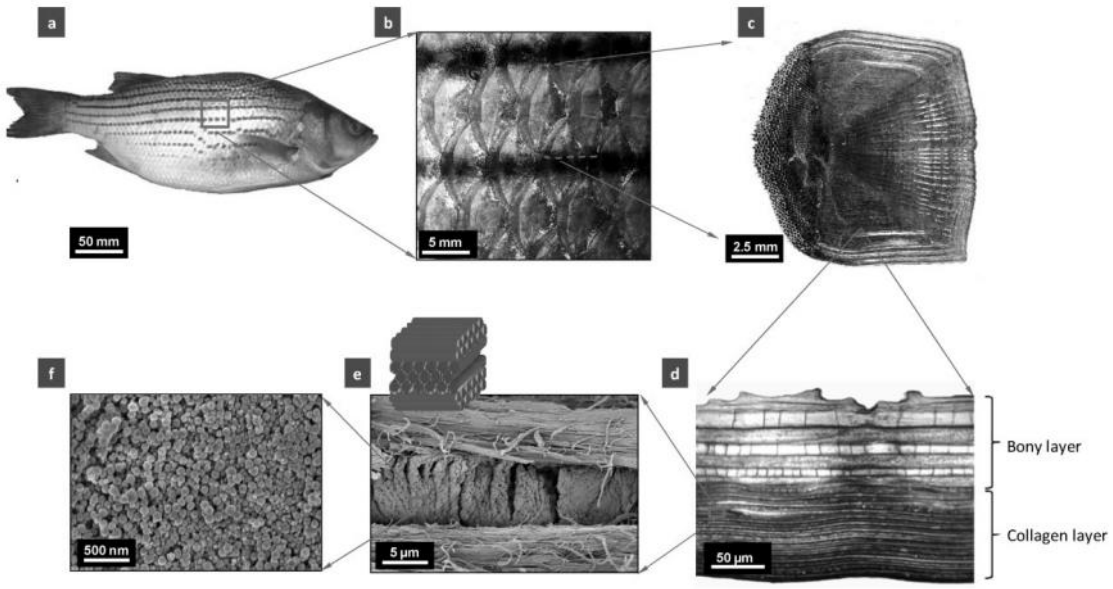

Figure 1.2 - The hierarchical structure of a fish scale: a) fish; b) staggered multiple scales; c) an individual scale; d) cross-section of a scale; e) cross-ply collagen structure; f) collagen fibrils (adapted from Zhu, D., et al. Structure and Mechanical Performance of a "Modern" Fish Scale. Advanced Engineering Materials. 2011)

At the macroscopic level, the scales are staggered together and cover most of the body of the fish, providing a form of armor plating to protect the fish from injury and disease transmission, and they also assist in swimming by reducing water friction and resistance [20].

The fish scales are composed of extracellular matrix, mainly type I collagen fibers and calcium-deficient hydroxyapatite (HAp), which together form a highly ordered three-dimensional structure. Each scale consists of two distinct regions: an osseous layer and a fibrillary plate mainly composed of collagen [21]. Then, fish scales are composed by HAp and type I collagen fibrils with a plywood structure of aligned fibril sheets. This fibrils alignment is similar to those of bones, tendons and corneas in the human body [22] [23]. Zhu et al. (2011) [19] studied the mechanical properties of fish scales and concluded that these are high performance natural protective systems considering the importance of the structure and architecture of the scales in "amplifying" the properties of their components.

Although the main sources of type I collagen are bovine or porcine dermis, outbreak of different transmissible diseases in pigs and cattle, restrict the use of collagens and allied products from these sources [24] [25] [26]. The collagen from the fish scales is unlikely to be associated with infections such as BSE, TSE and FMD [27] [28]. Collagen isolated from fish scales is already being studied for biomedical and pharmaceutical applications owing to its cell attachment capabilities, excellent biocompatibility, biodegradability and weak antigenicity [29]. Therefore, type I collagen derived from fish scales attracted much interest as an alternative collagen source for artificial substitutes and several authors already extracted collagen from fish scales and characterized it. Nagai et al. (2004) [25] reported that fish scale collagens were heterotrimers with chain composition of $(\alpha 1)_2$ and $\alpha 2$ and the denaturation temperature was lower than land animal collagen. Pati et al. (2010) [21] isolated collagen from Rohu and Catla and this collagen had a denaturation temperature of 36.5°C, which is promising as an advantage for biomedical application due to its closeness to mammalian collagen.

Fish scales from different species present variations in size, shape and arrangement. The general classification includes: placoid, cosmoid, ganoid, and elasmoid (cycloid and ctenoid). These elasmoid scales are more evolved scales when compared with the other types being thinner and more flexible, which improved the swimming capability, present greater hydrodynamic properties and a more resistant protective layer [19]. In this study we have investigated scales of the elasmoid cycloid type, which are a bit like tree trunks, because, as they age, they form concentric lines, which provide to scientists a way of determining the age of a fish.

The scales pattern allows also the establishment of a correspondence to events occurred in the life of a fish [30]. A cycloid scale is formed of a rigid surface layer consisting of calcium-based salts and a fibrous inner layer mainly made of collagen. Two different calcifying mechanisms function for a growing scale in the fibrillary plate and the osseous layer. Calcification in the osseous layer happens by nucleation of crystals with generation of larger patches, and in the fibrillary plate by uniform growth of crystals along the calcifying front [21].

Cells in tissues are arranged in distinct patterns; the orientation and the position of the cells with respect to each other are established by the tissue type. The study of the ability to control the placement of cells in an organized micro-pattern, such like the existent in the fish scales, on a substrate has become increasingly important for tissue engineering applications, which require that cells can be specifically placed to generate organized structures, such as a neural network for example [31] [32]. Also the ability to constrain the spreading to a specific cell-surface contact area has been shown to dramatically affect cellular development. Mechanical compliance of the cell-adhering substrates can also substantially affect the cells response and development [33]. Cells were reported to elongate in the direction of the micrometer-size grooves existent in the pattern and migrate as guided by these grooves, being this phenomenon denominated as contact guidance [34]. The disintegration and formation of fibrous cellular components is also influenced by surface topography. There is evidence that cell shape can control cell growth, gene expression, secretion of proteinases and ECM metabolism. Micro-patterned tissue engineered constructions are expected to better preserve cell morphology, differentiation and functionality for long periods of time. The micro-topography provides directional growth for cells and also can create tissue architecture at cellular and subcellular level in a reproducible manner [35].

Fish scales study is important in order to extract some principles for application in other fields, being the biomedicine a potential field for this application considering the fish scales composition, structure and functions. Another attractive reason to study the fish scales is the abundance and prosperity of its source and the fact that they are directed to low-added value ends. Marine capture fisheries mean over 50% of total world fish production and more than 70% of this production has been utilized for processing. As a result, every year considerable amounts of total catch are discarded as processing leftovers, consisting more than 30% of this waste of skin, scale and bone, which are rich in collagen and have received increasing attention as collagen sources. Recent estimates revealed that discards from the world's fisheries exceed 20

million tons, meaning 25% of the total production of marine capture fisheries. Therefore, there is a great potential in marine bioprocess industry to convert and utilize more of these products as valuable ones [36] [37] [38].

4 | Scaffolds Preparation with Natural Polymeric Materials

The main strategy of bone tissue engineering implicates the utilization of artificial extracellular matrixes as scaffolds in combination with specific types of cells under stimulation of growth factors in order to restore damaged bone both structurally and functionally. Biocompatibility and mechanical properties with suitable biodegradability are mandatory characteristics to be incorporated in these scaffolds. Matrices occurring naturally have advantages because of their relevant biocompatibility properties [7] [10] [13] [14] [39]. In this study fibroin and collagen films were prepared to be used as cell proliferation supports.

4.1. Fibroin

Silks are fibrous proteins commonly produced by insects and spiders. They exhibit a unique and useful conjunction of properties such as being degradable and lightweight with excellent mechanical and thermal properties. In nature, silks are used, for example, as materials for web construction and prey capture (spider webs), and reproduction enclosures (cocoons) where the silkworms are included. These features are also related to the current use of silks as sutures, nevertheless they are being gradually replaced by synthetic polymers. Silk consists of two different proteins: fibroin and sericin. Fibroin is the protein responsible for the filaments of silkworm silk and it can be regenerated in various ways depending on the application. Recently, researchers have investigated silk proteins, mainly silk fibroin, as one potential candidate material for biomedical applications, because it has several distinctive biological properties such as: significant biocompatibility, good oxygen and water vapor permeability, biodegradability, praiseworthy mechanical strength in the wet state, resistance against enzymatic cleavage, drug permeability and minimal inflammatory reaction [40] [41]. Beyond their traditional use as sutures, silk fibroin has been exploited as a biomaterial for cell culture and tissue engineering *in vitro* and *in vivo* since the mid-90s. In early studies, silk films obtained from native silkworm fibroin collected from glands of *Bombyx mori* domestic silkworms and *Antheraea pernyi* wild

silkworms were found to support the attachment and proliferation of fibroblasts. Later reports showed that regenerated silk films prepared by dissolution of silkworm cocoon fibers in 9–9.5 M LiBr supported the attachment and growth of human and animal cell lines. These studies provided clues to the use of regenerated silk fibroin as a scaffold or matrix biomaterial for cell culture and tissue engineering [40] [42] [43].

Numerous studies have already explored regenerated silk fibroin-based biomaterials in various forms, including films, membranes, mats, nets, hydrogels, and porous sponges for biomedical applications. The *in vivo* behavior of biomaterials prepared from silk fibroin solutions has not been completely demonstrated, despite the fact that the biocompatibility and degradation of silk sutures, which are prepared from native silk fibers, have been well established. Previous studies showed that two-dimensional silk fibroin films have good biocompatibility. Recent progress in processing techniques has yielded three-dimensional porous silk fibroin scaffolds with control of morphological and structural features [40] [42] [43].

4.2. Collagen

Collagen is the most abundant insoluble fibrous protein in the extracellular matrix and in the connective tissue. In fact, it is the major protein in vertebrates and in the animal kingdom, and constitutes about 25% of vertebrate total proteins and approximately 30% of all protein in the human body. There are at least 16 types of collagen, but 80-90% of the collagen in the human body consists of types I, II, and III. These collagen molecules pack together forming long thin *fibrils* with similar structure. In contrast, type IV forms a two-dimensional reticulum. Other types of collagen can associate with fibril-type collagens, link between each other or link with other matrix components. Firstly it was thought that collagens were secreted by fibroblasts in connective tissue, but is now known that numerous epithelial cells produce certain types of collagen. The various collagens existent and the structures they form have the same purpose: to help tissues resist stretching [44].

The collagen fundamental structural unit is a long (300 nm), thin (1.5 nm of diameter) protein that consists of three coiled subunits: two $\alpha 1$ chains and one $\alpha 2$. Each chain consists precisely of 1050 amino acids wounded around one another in a typical right-handed triple helix [45] [46].

Type I collagen fibrils have a immense tensile strength, which means that such fibrils can be highly stretched without being broken. These fibrils, approximately 50 nm in diameter and several micrometers long, are packed side-by-side in parallel bundles, denominated *collagen fibers,* which can be found for example in tendons, where they connect muscles with bones and have to withstand high tension [44] [47].

In tissue engineering, collagen is being used for development of scaffolds either alone or in combination with other biomaterials for skin replacement, as artificial blood vessel, heart valve, bone graft, cartilage and ligament replacement among others. The utilization of collagen in pharmaceutical applications includes production of wound dressings, vitreous implants and as carriers for drug delivery. The applications of collagen in drug delivery systems are quite diverse such as collagen shields in ophthalmology, sponges for burns/wounds, mini-pellets and tablets for protein delivery, gel formulation in combination with liposomes for sustained drug delivery, as controlling material for transdermal delivery, and nanoparticles for gene delivery. In addition, collagen is used as surgical sutures and as haemostatic agent [29] [48] [49] .

There are many attractive characteristics that make the collagen type I a popular ECM of connective tissue. Some examples are: biocompatible, osteocompatible, fibrous, adhesive, cohesive, nonfriable, suturable, highly porous, can be combined with other materials, etc. The main limitation is the inexistence of inherent rigidity [39] [50]. To optimize this mechanical property and modify the biodegrading rate, an effective method commonly used is the cross-linking of the collagen or the addition of ceramics, such as hydroxyapatite [51].

5 | Cell Types

As previously referred, in bone tissue engineering, cells play a fundamental role for the bone regeneration. For that reason, the utilization of cells with the ability and potential for osteogenesis is mandatory.

5.1. Osteoblasts

Osteoblasts are cells of mesenchymal origin responsible for synthesis and mineralization of bone during both bone formation and bone remodeling. They form a closely packed sheet on the bone's surface, from which cellular processes extend through the developing bone. The

goals for scaffolds in bone tissue engineering include suitable chemistry, morphology and structure in order to promote cell adhesion for osteoblasts, their further migration, and synthesis of extracellular matrix and mineralization [52] [53] [3].

The osteoblasts form organic, non-mineralized bone matrix and are involved in complex interactions with a variety of factors, mediators and cell types [54]. Mature osteoblasts are highly polarized, and present a prominent Golgi apparatus which is typical of highly secretory cells. The main secretory product of osteoblasts is type I collagen [55].

5.2. Adipose-derived Stem Cells

Tissue engineering and regenerative medicine are multidisciplinary sciences evolved in parallel with recent biotechnological advances. It combines biomaterials, growth factors, and stem cells to repair failing organs. Stem cells are characterized by their capability to self-renew and their ability to differentiate along multiple lineage pathways. Among the several types, human subcutaneous adipose tissue emerges as a source of adult or somatic stem cells [56].

Due to the increased incidence of obesity all over the world, subcutaneous adipose tissue is abundant and readily accessible. For example, approximately 400,000 liposuction surgeries are performed in the United States of America every year. These procedures can yield from 100mL to 3L of lipoaspirate tissue. This resultant material is routinely discarded [57].

Recent developments in the field of stem cells suggested that hASCs can be induced into adipogenic, osteogenic, chondrogenic, and myogenic lineages under appropriate conditions. hASCs are easy to obtain, have relatively lower donor site morbidity and a higher yield at harvest, and can expand more rapidly *in vitro* compared with BMSCs for example, which are now commonly used as seed cells for bone repair and regeneration. In addition, hASCs have multipotency which is independent of serum source and quality. Thus, hASCs might be a novel and very promising alternative cell source for bone tissue engineering [13].

6 | Concluding Remarks

Bone diseases are variable and can affect people in different levels. The existent treatments are, in many cases, not enough for good recovery of the patient. There is still much

room for development of more effective treatment strategies and bone tissue engineering can provide promising alternatives for more efficient treatments.

Bone tissue engineering has a long path to be explored and the definition of the best strategies to achieve better results is still not clear. Therefore, attempts to find different alternatives are important, being the study of the effect of fish scales pattern on osteoblasts and stem cells the testing of hypothesis that can extend the knowledge of cells behavior when submitted to different environments.

Fish scales present interesting characteristics attractive for their study as cellular supports, such as being highly composed of collagen and hidroxyapatite and presenting a very organized structure.

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CHAPTER II.

MATERIALS AND METHODS

1 | Fish Scales Preparation

The fish scales to be studied were collected from a market in Thailand. The studied fish specie was *Lates Calcarifer*, being the common name White Seabass.

The scales preparation procedures were adapted from Ikoma T. et al [26] and Pati F. et al [5]. In brief, the scales were immersed in distilled water and conserved at 4°C. A group of scales was stored in an antibacterial solution of sodium azide 1%wt for further investigation of this group of untreated fish scales (G1). The remaining scales were washed twice in 10%wt of NaCl solution, to remove unnecessary proteins on the surface, for 48h. Afterwards, one part of these scales was washed with distilled water forming a second group (G2), and the remaining scales were submitted to a process of demineralization and extraction of the soluble collagen, in order to achieve the third group of scales (G3). For the demineralization, it was used a solution of 0.5 mol/L of EDTA, to immerse the fish scales for 48h. Then the extraction of soluble collagen was done by the immersion of the scales in a solution of 0.5 mol/L of Tris, HCl buffered at pH 7.5, for 48h. Scales were finally washed with distilled water and kept in it until further use.

2 | Fish Scales Characterization

2.1. Morphology

The scales of the 3 groups were observed in the Optical Microscope (Axiotech, Zeiss) and in the SEM (Supra 40, Zeiss) in order to analyze the surface patterns. They were also observed in the Polarized Microscope and in the Confocal Laser Microscope (Eclipse, Ti-E, Nikon).

2.2. Collagen Imaging

Confocal Laser Microscope (Eclipse, Ti-E, Nikon) was used to evaluate collagen architecture on different group samples, and Direct Red 80 [27] was used as unspecific stain for collagen, typically used for detection in tissue histological section. Samples were stained for 60 minutes in an aqueous solution of 0.1% of Sirius Red F3BA (Sigma Aldrich) saturated with picric acid, pH 2. After that, samples were washed for 2 minutes in 0.01M HCl, rinsed in ethanol 70%, and dehydrated with ethanol gradients, 70, 90, and 2x100%.

3 | Fibroin Films

3.1. Fibroin Production

In addition to G1, G2 and G3 groups, two more groups were created. Fibroin films with the same patterning as the scales (F1), and fibroin films without patterning (F2), in order to allow drawing conclusions about the pattern role in the cellular behavior.

In order to mimic the fish scales pattern, the material selected was the fibroin. To prepare the fibroin needed, the process was divided in two steps: degumming and dissolution.

Degumming

Bombyx mori cocoons (kindly provided by Socio Lario, Cassina Rizzardi, Como, Italy) were degummed for 1.5 hours in a bath of boiling water containing 1.1 g/l Na_2CO_3 (10 g of silk in 1L of solution), then for 1.5 hours in another bath of boiling water containing 0.4 g/l Na_2CO_3. After the two baths cocoons were rinsed thoroughly with distilled warm water to remove salts and dried in air.

Dissolution

Fibroin-water solutions were prepared by dissolving the fibroin obtained in the previous step in 9.3 M LiBr (Fluka Chemical) aqueous solution (1g/10ml) at 65°C for 2 hours, followed by dialysis against distilled water with a 3500 Da MWCO membrane (Slyde-A-Lyzer, Pierce) in order to eliminate salts.

The concentration of the resultant fibroin solution was measured by UV-VIS spectrophotometer (Nanodrop). The solution was then diluted to a concentration of 2.5% w/v. The pH of the solution was 5.

3.2. Construction of fibroin samples with the fish scales pattern (F1)

The first step of this process was the creation of silicon molds with the fish scales pattern. This was achieved using a plate with nine prominent cylinders with a glued fish scale with the pattern turned up on the top of each cylinder, as shown in figure 2.1.

Figure 2.1 - Plate where the scales were glued and covered with silicon to form the molds with scale patterning

The plate was filled with a silicon solution (Sylgard Silicone Elastomer kit 184) and the air bubbles were removed with the aid of a dissector. Then the plate was transferred to an oven at 65°C during 24h. The resultant molds contained negatives of fish scales patterns. The result of this process is shown in figure 2.2, as the bottom of each of its wells transducers the negative of the fish scales pattern.

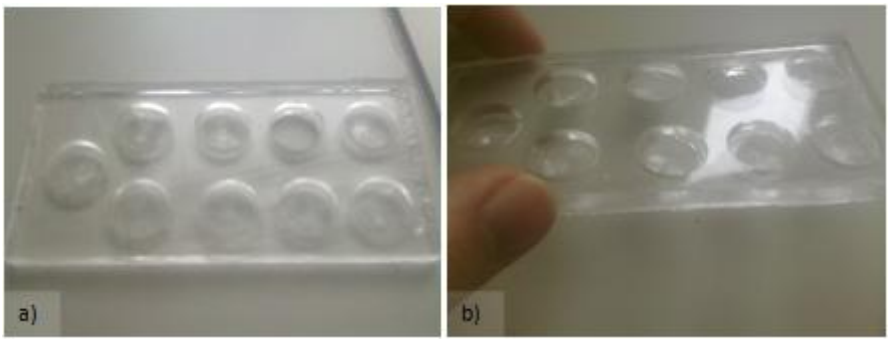

Figure 2.2 - a), b) - Silicon mold with the negative of the fish scales pattern in the bottom of each well

Figure 2.3 refers to the pattern seen in one of the resultant negatives using the Optical Microscope (Axiotech, Zeiss), being similar to the fish scales pattern, as it will be shown further ahead.

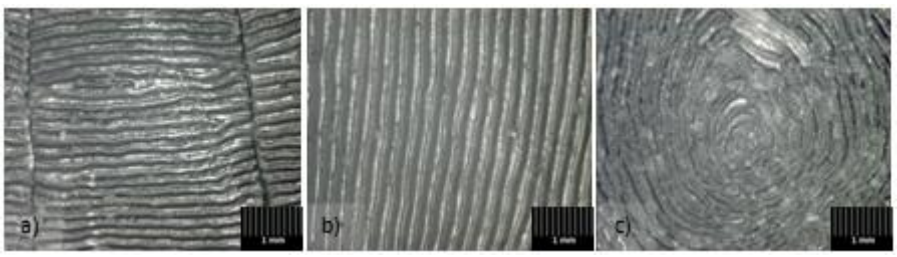

Figure 2.3 - a), b), c) - Pattern of the negatives existent in the bottom of each well of the silicon mold

To achieve the copy of the pattern of the fish scales, the wells containing the negatives were filled with the fibroin solution and left drying for 3 days at room temperature. From each well, a sample of fibroin like the one shown in figure 2.4 was produced.

Figure 2.4 - Fibroin sample with a pattern similar to the fish scales

Figure 2.5 shows the pattern of one of the resultant samples of fibroin observed using the Optical Microscope, similar to the fish scales, as shown.

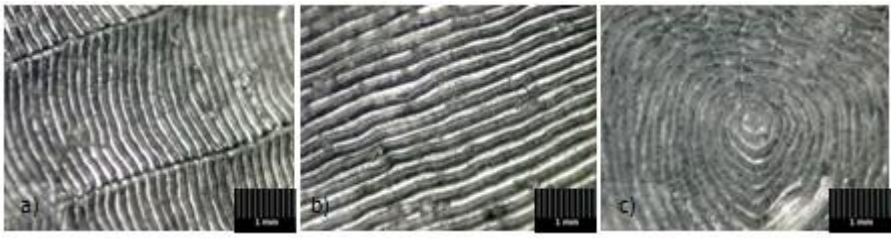

Figure 2.5 - a), b), c) - Fibroin sample pattern observed in the Optical Microscope

In order to stabilize the fibroin samples, these were immersed in an aqueous solution of methanol 80% for 20 minutes, and then washed with distilled water. This procedure was shown not to affect the pattern.

3.3. Construction of the fibroin samples without pattern (F2)

Fibroin films were prepared by casting aqueous solutions with 2.5% w/v of total protein content on polyethylene plates at room temperature for 2 days. After that, the film was cut in circles with controlled diameter. All resulting films were stabilized using the same procedure as the samples with the scale pattern.

Figure 2.6 - Fibroin sample without any pattern

4 | Collagen films

4.1. Extraction of collagen from fish scales

In a first phase the idea was to construct collagen films with collagen directly extracted from fish scales. Fish scales were collected from the company *Ramirez*. These scales were from the fish kind Sardina pilchardus, being the common name Sardine.

The scales were preserved in the freezer at -80°C till their utilization. At this time they were washed several times with water and then completely dried with the aid of a liophilizer in order to measure their weight. The Sardine fish scales were treated with 10 % NaCl solution during 48h to remove unnecessary proteins. Then, they were demineralized by immersion in 0.5 mol/L EDTA solution during 48h. To extract the collagen from the scales, these were immersed in 0,5mol/L Tris-HCl solution, buffered at pH 7.5, at 4 °C, during 48 hours. The resultant suspension was centrifuged at 4°C and 10,000 x g for 20 minutes to separate the collagen. The resultant residue of this process was dissolved in 0,01M HCl solution. After 48 hours, the collagen was precipitated from the acid solution by increasing solution pH to 7 by addition of NaOH solution. The acid soluble collagen was then separated by filtration and washed with distilled water. The collagen was further purified by dissolution in 0.5M acetic acid, dialized against 0.1M acetic acid during 2 days.

For the quantification of the collagen existent in the solution after the dialysis was executed a method named as *SIRCOL*. Firstly was labeled a set of microcentrifuge eppendorfs. Then was prepared a standard curve, being the blanks constituted of 100µL of acetic acid 0,1M and the collagen standards (5, 10, 20, 30, 40µg) and making 100µL by adding acetic acid 0,1M

and finally vortexing these mixtures. The test samples were done in duplicate having each sample 100µL too. To each eppendorf created was added 1mL of Sircol Dye reagent. The eppendorfs were placed in a mechanical shaker for 30 minutes for the binding of the Sircol dye with soluble collagen. The eppendorfs were transferred to a micro centrifuge and spin at 13.000g for 10 minutes. The unbound dye solution was removed by carefully inverting and draining the tubes. Remaining droplets were removed by gently taping the inverted eppendorf. To each eppendorf was added 1mL of the Alkali reagent and the bound dye was released into solution using a vortex. After dissolution, the samples absorbance was measured using the Spectrophotometer set to a wavelength of 540nm. This data was collected and used to infer about the collagen existent by comparing the obtained values for the samples with the obtained standard curve. The yield of collagen extracted was very low, being about 0.01% on dry weight basis.

As an attempt to raise the efficiency of the process of collagen extraction, to the previous procedure were changed some parameters. This time, the whole procedure was executed at a temperature of 4°C; the scales while immersed in the solutions for unnecessary proteins removal, demineralization and collagen extraction were submitted to constant agitation; the suspensions centrifugation was done at 20,000 x g for 1 hour; and the dialysis was done against 0,5M acetic acid instead of 0,1M. This attempt succeeded with a raise in the yield of collagen extracted, being this time of about 0.2% on dry weight basis. Although this method was still very inefficient, even after executing this procedure five times the collagen collected was still in a very low quantity for the application it was needed – the production of collagen films.

After this, the encountered way was to produce the collagen films with already available collagen and with still fish origins, being the solution the production of the films based on shark skin collagen.

4.2. Construction of shark collagen films with scales patterning

The shark skin collagen used was kindly provided by Ricardo I. Perez-Martin and Carmen G. Sotelo (CSIC, Inst Invest Marinas, Vigo 36208, Galicia, Spain). For the production of the films the collagen concentration used was 4% dissolved in acetic acid 0,5M. For the formation of stable films was then needed the cross-linking of collagen. For the cross-linking the first tries were with EDC and NHS but these were somehow frustrated because the results were always a gelatin, being unstable films. The solution found was the utilization of Genipin for the cross-linking. For

that, Genipin was added to the collagen solution in order to be in a concentration of 10mM. Then, while stirring, the solution was inserted in a mineral oil bath at 37°C and controlled while was getting more and more viscous and changing to a darker color. After about 3h of this process the solution was already darker and more viscous and was transferred to proper recipients.

The shark skin collagen films with the fish scales pattern were achieved with the utilization of silicon molds with wells with the scales pattern which were constructed with a similar method as the one described in section 2.1.2. Each well of these molds was filled with 1mL of the collagen solution prepared and after 3 days were formed stable dry collagen films with the fish scales pattern.

5 | In vitro Biological tests

The experiment *in vitro* described below was preceded by a simpler *in vitro* experiment. This experiment studied the cellular behavior (osteoblasts) when seeded on the fish scales from the 3 groups for 5 days. Promising results were achieved by the execution of the Alamar Blue tests, Confocal Microscope and SEM analysis.

5.1. Cells culture and seeding

Before the *in vitro* tests, the scales (G1, G2, G3) and fibroin films (F1, F2) were immersed in ethanol 70% at 4°C respectively for 24 hours and 2 hours and washed with sterile distilled water.

Osteoblasts of MG63 cell line were cultured in Minimum Essential Medium (MEM) (Invitrogen), 10% Fetal Calf Serum, 1% Sodium Piruvate, 100 mM Euroclone, 2% L-glutamine 100 mM Euroclone, Non Essential Aminoacids Gibco, and 1% of Antibiotic / Antimicotic. Cells were cultured in an atmosphere of 37°C and 5% CO_2. After the cells have reached confluence, they were detached by the removal of the medium, the washing of the flask with PBS and the addition of trypsin. The cell concentration was $1{,}0x10^5$ cells/ml. To execute this protocol, 37 samples from each group (G1, G2, G3, F1 and F2) were used. After 1 day, 4 samples of each group were removed from the experiment to be analyzed (2 by SEM and 2 by Confocal Microscope), and another 4 samples from each group were removed for the ALP test. The same was done after 3,

7 and 14 days, when the experiment was concluded. It is important to mention that the samples for the Alamar Blue test were always the same. This means that 5 samples from each group were used during the whole experiment, since this procedure does not damage the cells. The *in vitro* experiment had the duration of 14 days: day 0 was considered the day of seeding; on day 1 all the samples were switched to different well plates in order to remove all the cells not attached to the samples from the equation, as well as it was the first day of control; day 3 was the second day of control; on day 5, a change of the medium was done; day 7 was the third day of control; on days 9 and 11, a change of medium was done; and finally day 14 was the last day of control. On the days of control, the Alamar Blue test was performed and the samples to be controlled in that day were submitted to a protocol of fixation.

The potential of fish scales to support the osteogenic differentiation of human adipose derived stem cells (hASCs) was studied. Scales from *Lates calcarifer* were submitted to a treatment for removal of unnecessary proteins on their surface by immersion in NaCl (Panreac) 10%wt during 48h, equivalent to the already mentioned for the G2 samples. All the scales were cut in circles with 10mm of diameter and immersed in ethanol 70% during 2 hours for sterilization. After this, scales were washed with sterile PBS. Cells were cultured in basal culture medium (α-MEM plus 10% FBS and 1% of A/B) and standard osteogenic medium (α-MEM plus 10% FBS and 1% of A/B, and supplemented with 10 mM beta-glycerophosphate, 50 g/mL of ascorbic acid and 10-8M of dexamethasone). hASCs at passage 2 were seeded at a density of 2000 cells/cm^2. The quantity of medium immersing each sample was of 1ml. Tissue culture polystyrene coverslips (TCPs) were used as controls. Cultures were maintained for 1, 3, 10, 21 and 30 days.

5.2. AlamarBlue Assay

AlamarBlue assay kit (Biosource International Inc., USA) was used to assess metabolic activity at the end of each experimental time point of the studies with MG63. This technique is a quantitative process providing a linear measurement over time, being safe and nontoxic for the cells. It is designed to measure the proliferation of various human and animal cell lines quantitatively. A higher proliferation causes a larger absorbance value as well as a larger percentage of reduced alamarBlue. The assay was performed according to the manufacturer's instructions [4].

In brief, the seeded samples were incubated for 4 hours at 37°C with fresh culture medium (with reduced serum) supplemented with alamarBlue diluted according to manufacturer's data sheet (simply adding the alamarBlue reagent as 10% of the sample volume). A total of 3 replicates were used for each sample and each replicate was split into 4 wells for the final reading. The references were taken from wells with unseeded samples incubated with the alamarBlue solution, wells with only culture medium, and wells with only cells. Absorbance was measured at 570 nm and 620 nm with a photometric microplate reader (Multiskan EX, ThermoLabsystems, Finland) and the percentage of reduced alamarBlue was calculated. The whole procedure was executed in the absence of light.

The calculations to get the percentage of reduced alamarBlue (being this percentage directly proportional to the quantity of cells existent) were:

- Subtract the absorbance values of medium only from the absorbance values of Alamar Blue.
- Calling AO_{LW} = absorbance of form at lower wavelength, and AO_{HW} = absorbance of oxidized form at higher wavelength, correction factor: $R_0 = AO_{LW} / AO_{HW}$.
- To calculate the percentage of reduced Alamar Blue in a specific well: $\%Reduced = A_{LW} - (A_{HW} \times R_0) \times 100$, being A_{LW} the absorbance at 570nm and A_{HW} the absorbance at 620nm.

Statistical Analysis

Statistical Analysis was performed for the AlamarBlue assay results which are presented as means ± STDEV (Standard Deviation of the Mean). Friedman Test (non- parametric test equivalent to One Way Repeated Measurements ANOVA) was performed for each group analyzed along time. One Way ANOVA on Ranks followed by Tukey Test was performed for each time point for group comparison (SigmaStat 3.0, SPSS, Chicago, IL, USA).

5.3. Confocal Laser Microscope (CLM) Analysis

Evaluation of cell attachment, distribution, and morphology on fish scales (G1, G2 and G3) and fibroin films (F1 and F2) seeded with MG63 was performed by confocal laser microscopy (Nikon Eclipse, Ti-E) after Phalloidin Rhodamine (Biosource International, Invitrogen) and DAPI (Sigma Aldrich) staining according to the manufacturer's protocol. Fixation with a

formaldehyde solution (4% formaldehyde in PBS solution) and permeabilization with TritonX 100 (0.2% TritonX in PBS solution) were performed before staining.

5.4. Scanning Electron Microscope (SEM) Analysis

Morphological observations were performed with scanning electron microscopy (Supra 40 Zeiss), operating mode: high vacuum, secondary electron detector) on fish scales (G1, G2 and G3) and fibroin films (F1 and F2) seeded with MG63. At each time point, films were fixed with gluteraldehyde solution (2,5% gluteraldehyde in cacodylic buffer solution, 0,1 M) to preserve biological structures. Samples dehydration was performed by soaking in a series of aqueous ethanol solutions at increasing concentrations from 30% - 100% and drying in air at room temperature. Before SEM imaging, all samples were sputter coated with gold (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy).

5.5. Alkaline Phosphatase (ALP) activity quantification

Alkaline Phosphatase (ALP) is the biochemical marker of osteoblastic bone formation most frequently used. The activity of ALP is evaluated using p-nitrophenol assay. Para-nitrophenyl phosphate, which is colorless, is hydrolysed by alkaline phosphatase at 37°C to form free paranitrophenol, which is colored yellow. The reaction is stopped by addition of NaOH and the absorbance read at 405 nm.

Osteoblastic activity on fish scales (G1, G2 and G3) and fibroin fims (F1 and F2) was monitored measuring Alkaline phosphatase activity (ALP) based on the conversion of p-nitrophenyl phosphate to p-nitrophenol. Firstly, solutions were added in nine test tubes (Sample Diluent, Buffer Solution and p-Nitrophenol Standard) in order to build a standard curve, as shown in table 2.1.

Tube #	1	2	3	4	5	6	7	8	9
Sample Diluent (µL)	250	250	250	250	250	250	250	250	250
Buffer Solution (µL)	250	245	240	230	220	210	200	190	180
pNP Standard (µL)	0	5	10	20	30	40	50	60	70
Concentration (µmol/mL)	0	1	2	4	6	8	10	12	14

Table 2.1 - Solutions execution for the nine test tubes in order to build the standard curve for ALP test (osteoblasts)

All the tubes were vortexed and then 100µl from each standard tube were transferred to a 96-well plate. After this, the same quantity of NaOH solution was added to all the standard

wells and measured spectrophotometrically at 405 nm in a microplate reader (Multiskan EX, ThermoLabsystems, Finland), to set up the calibration curve.

To prepare the samples reading, 50µl from each sample (all the samples in quadruplicate) were put in a 96-well plate, 50µl of 0.05% Triton-X (Sigma) in PBS were put in 6 wells to use as blank, and then 50µl of Buffer Solution were added. Finally the samples were incubated at 37°C till the majority of the solutions in the wells turned yellow, which happened after 3h20mins for the samples from days 1 and 3 of control, and after 1h42mins for the samples from days 7 and 14 of control.

This procedure presented some differences when executed for the scales of G2 seeded with hASCs. The following solutions were used: substrate buffer (1M Diethanolamine HCl, pH9.8 - Sigma); substrate solution (0.2% w/v p-nytrophenyl phosphate in substrate buffer); stop solution and blank (2M NaOH/0.2mM EDTA); product/standard solution (p-nytrophenol stock solution 10µmol/ml – Sigma); and PBS.

In each well of 96-well plates the following mixture was made: 20µl of sample and 60µl of substrate in triplicate. Then the 96-well plates were incubated at 37°C for 45min. After the incubation period, 80µl of stop solution were added to each well. The ALP standards were prepared according to table 2.2:

pNP Stock Solution (µl)	0.0	0.8	1.6	2.4	3.2
Stop Solution (µl)	160	159.2	158.4	157.6	156.8
Standard Concentration (µmol/ml)	0.00	0.05	0.10	0.15	0.20

Table 2.2 - Solutions execution for the nine test tubes in order to build the standard curve for ALP test (stem cells)

5.6. Macro BCA Protein Assay

In order to find the protein concentration of all samples, the Macro BCA Protein assay (micro BCA protein assay kit, Thermo Scientific, USA) was performed. In nine test tubes, the standard solutions were prepared, using different concentrations of Sample Diluent and Albumin Standard in each tube, as shown in table 2.3.

Tube #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample Diluent (µL)	200	195	190	180	160	140	120	100	80
Albumin Standard (µL)	0	5	10	20	40	60	80	100	120
Concentration (µg/µL)	0	0.05	0.1	0.2	0.4	0.6	0.8	1	1.2

Table 2.3 - Solutions execution for the nine test tubes in order to build the standard curve for BCA assay

All the tubes were then vortexed and 25µl were transferred in triplicate to a 96-well plate, in order to measure the absorbance and create the calibration curve.

To prepare the samples absorbance reading, 25µl from each one were transferred in duplicate to a 96-well plate. Another 6 wells were used as blank, putting 25µl of Sample Diluent in each one. Then, to each well, including standards, samples and blanks, 200µl of working solution was added (prepared with two different reagents as indicated in protocol). Finally the samples were incubated at 37°C for 30 minutes and read in the microplate reader (Multiskan EX, ThermoLabsystems, Finland) at 570nm.

5.7. DNA measurement

DNA content in fish scales (G1, G2 and G3) and fibroin fims (F1 and F2) seeded with MG63 was measured through Picogreen, according to the manufacturer's protocol (Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Firstly, the preparation of TE solution and a dilute PicoGreen solution following a protocol (Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) was performed. With the prepared solutions, eight assay tubes were filled with different quantities in order to set the standard solutions. To the wells of a 96-well plate, 100µl of the standards and samples in duplicate were transferred, and 100µl of PicoGreen solution were added. Finally, the plate was read on a fluorescent plate reader, exciting at 485nm and reading at 538nm.

The procedure executed for the DNA quantification for the scales of G2 seeded with hASCs presented some differences. After the samples defrosting and the preparation of the necessary solutions following the previously mentioned indications of the assay kit manufacturer, in each well of 96-well white opaque plates the following mixture was made: 28.7µl of sample or standard, 71.3µl of *Picogreen* solution and 100µl of 1XTE. Then the plates were incubated in the dark for 10min and the fluorescence read using the parameters of excitation of 485nm and emission of 528nm.

5.8. Fluorescence Microscopy Analysis

Evaluation of stem cell attachment and distribution on the seeded samples was performed by fluorescence microscopy using a Zeiss Axioimager Z1 microscope (Zeiss, Germany) after Phalloidin Rhodamine (Sigma) and DAPI (Sigma) staining according to the manufacturer's protocol. Fixation with Formalin 10% (BioOptica) was performed before staining.

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CHAPTER III.

FISH SCALES AS MODEL FOR OSTEOGENIC CELLS CULTURE

Fish Scales as Model for Osteogenic Cells Culture

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Abstract

"Biomimetics" refers to human-made substances, processes, devices, or systems that somehow imitate nature. Creatures and plants possess tools and skills that are attractive to imitate, being the goal to learn its principles, and thereafter transfer the principle to a synthetic process or new material. Considering the fish scales composition of hydroxyapatite and type I collagen fibrils, and also their capability to provide a form of armor plating to protect it from injury and disease transmission. The fish scales study will allow us to extract some principles for application in other fields, being the biomedicine a potential field for this application. In the present work, fish scales of the specie *Lates Calcarifer*, also known as White Seabass, were studied. Scales characterization was done by observations in the Optical Microscope, SEM and Confocal Laser Microscope, with specific focus on their pattern. Fish scales were found to be very organized consisting mainly of parallel concentric grooved lines. *In vitro* biological analysis of the scales was performed using them as patterned films for cell culture. Scales supported cell proliferation and the cells presented an orientation according to the fish scales patterning

alignment both for osteoblasts (MG63) and hASCs, acquiring an elongated and narrow shape. The present work is the first to evaluate cell behavior induced by fish scale patterning.

1 | Introduction

"Biomimetics" is a recent definition but in fact the humankind was inspired by Nature since the beginning of civilization. Creatures and plants possess tools and skills that are attractive to mimic. Mimicking nature cannot be a goal in itself, once this would hardly represent progress. The goal is to learn its principles, extract the general physic-chemical processes of the object/principle to be mimicked, and thereafter transfer it to a synthetic process or new material [1]. Fish scales study is relevant in order to extract some principles for appliance in different areas, being the biomedicine a potential *environment* for this praxis considering the fish scales composition, structure and functions.

This study focuses on the fish scales of a specie denominated *Lates Calcarifer*, which is one of the nine *Lates* species of the family *Centropomidae* [2] [3].

At the macroscopic level, fish scales are staggered together and cover most of the body of the fish, providing a form of armor plating to protect the fish from injury and disease transmission, and they also assist in swimming by reducing water friction and resistance [4].

The fish scales are composed of extracellular matrix, mainly type I collagen fibers and calcium-deficient hydroxyapatite (HAp), which together form a highly ordered three-dimensional structure. Each scale consists of two distinct regions: an osseous layer and a fibrillary plate mainly composed of collagen [5]. Fish scales are composed by HAp and type I collagen fibrils with a plywood structure of aligned fibril sheets. This fibrils alignment is similar to those of bones, tendons and corneas in the human body [6] [7]. Zhu et al. (2011) [8] studied the mechanical properties of fish scales and concluded that these are high performance natural protective systems, considering the importance of the structure and architecture of the scales in amplifying the properties of their components.

Fish scales from different species present variations in size, shape and arrangement. The general classification includes: placoid, cosmoid, ganoid, and elasmoid (cycloid and ctenoid). These elasmoid scales are more evolved scales when compared with the other types being thinner and more flexible, which improved the swimming capability, present greater

hydrodynamic properties and a more resistant protective layer [8]. In this study we have investigated scales of the elasmoid cycloid type, which are similar to tree trunks, because, as they age, they form concentric lines, which provide to scientists a way of determining the age of a fish. The scales pattern allows also the establishment of a correspondence to events occurred in the life of a fish [9].

Cells in tissues are arranged in distinct patterns; the orientation and the position of the cells with respect to each other are established by the tissue type. The study of the ability to control the placement of cells in an organized micro-pattern, such like the existent in the fish scales, on a substrate has become increasingly important for tissue engineering applications, which require that cells can be specifically placed to generate organized structures, such as a neural network for example [10] [11]. Also the ability to constrain the spreading to a specific cell-surface contact area has been shown to dramatically affect cellular development. Mechanical compliance of the cell-adhering substrates can also substantially affect the cells response and development [12]. Cells were reported to elongate in the direction of the micrometer-size grooves existing in the pattern and migrate as guided by these grooves. The disintegration and formation of fibrous cellular components is also influenced by surface topography. There is evidence that cell shape can control cell growth, gene expression, secretion of proteinases and ECM metabolism. Micro-patterned tissue engineered constructions are expected to better preserve cell morphology, differentiation and functionality for long periods of time. The micro-topography provides directional growth for cells and also can create tissue architecture at cellular and subcellular level in a reproducible manner [13].

Another attractive reason to study the fish scales is the abundance and prosperity of its source and the fact that they are directed to low-added value ends. Marine capture fisheries represent over 50% of total world fish production and more than 70% of this production has been utilized for fish processing. As a result, every year considerable amounts of total catch are discarded as processing leftovers, consisting more than 30% of this waste of skin, scale and bone, which are rich in collagen and have received increasing attention as collagen sources. Recent estimates revealed that discards from the world's fisheries exceed 20 million tons, meaning 25% of the total production of marine capture fisheries. Therefore, there is a great potential in marine bioprocess industry to convert and utilize more of these products as added valuable products [14] [15] [16].

Silks are fibrous proteins commonly produced by insects and spiders. They exhibit a unique and useful conjunction of properties such as being degradable and lightweight with excellent mechanical and thermal properties. In nature, silks are used, for example, as materials for web construction and prey capture (spider webs), and reproduction enclosures (cocoons) where the silkworms are included. These features are also related to the current use of silks as sutures, nevertheless they are being gradually replaced by synthetic polymers. Silk consists of two different proteins: fibroin and sericin. Fibroin is the protein responsible for the filaments of silkworm silk and it can be regenerated in various ways depending on the application. Recently, many researchers have investigated silk proteins, mainly silk fibroin, as one of the candidate materials for biomedical applications, because it has several distinctive biological properties such as: significant biocompatibility, good oxygen and water vapor permeability, biodegradability, praiseworthy mechanical strength in the wet state, resistance against enzymatic cleavage, drug permeability and minimal inflammatory reaction [17] [18]. Beyond their traditional use as sutures, silk fibroin has been exploited as a biomaterial for cell culture and tissue engineering *in vitro* and *in vivo* since the mid-90s. In early studies, silk films obtained from native silkworm fibroin collected from glands of *Bombyx mori* domestic silkworms and *Antheraea pernyi* wild silkworms were found to support the attachment and proliferation of fibroblasts. Later reports showed that regenerated silk films prepared by dissolution of silkworm cocoon fibers in 9–9.5 M LiBr supported the attachment and growth of human and animal cell lines. These studies provided clues to the use of regenerated silk fibroin as a scaffold or matrix biomaterial for cell culture and tissue engineering. Numerous studies have already explored regenerated silk fibroin-based biomaterials in various forms, including films, membranes, mats, nets, hydrogels, and porous sponges for biomedical applications. The *in vivo* behavior of biomaterials prepared from silk fibroin solutions has not been completely demonstrated, despite the fact that the biocompatibility and degradation of silk sutures, which are prepared from native silk fibers, have been well established. Previous studies showed that two-dimensional silk fibroin films have good biocompatibility. Recent progress in processing techniques has yielded three-dimensional porous silk fibroin scaffolds with control of morphological and structural features [17] [19] [20].

Osteoblasts are cells of mesenchymal origin responsible for synthesis and mineralization of bone during both bone formation and bone remodeling. They form a closely packed sheet on the bone's surface, from which cellular processes extend through the developing bone. The goals for scaffolds in bone tissue engineering include suitable chemistry, morphology and

structure in order to promote cell adhesion for osteoblasts, migration, differentiation, synthesis of extracellular matrix and mineralization [21] [22] [23].

Stem cells are characterized by their capability to self-renew and their ability to differentiate along multiple lineage pathways. Human subcutaneous adipose tissue emerges as a source of adult or somatic stem cells [24]. Recent developments in the field of stem cells suggested that hASCs can be induced into adipogenic, osteogenic, chondrogenic, and myogenic lineages under appropriate conditions. hASCs are easy to obtain, have relatively lower donor site morbidity and a higher yield at harvest, and can expand more rapidly *in vitro* compared with BMSCs for example, which are now commonly used as seed cells for bone repair and regeneration. In addition, hASCs have multipotency which is independent of serum source and quality. Thus, hASCs might be a novel and very promising alternative cell source for bone tissue engineering [25].

Concluding, the main objective of this study was determining both osteoblasts and stem cells behavior when submitted to the fish scales pattern.

2 | Materials and Methods

2.1. Fish scales preparation

The fish scales to be studied were collected from a market in Thailand. The studied fish specie was *Lates Calcarifer*, being the common name White Seabass.

The scales preparation procedures were adapted from Ikoma T. et al [26] and Pati F. et al [5]. In brief, the scales were immersed in distilled water and conserved at 4°C. A group of scales was stored in an antibacterial solution of sodium azide 1%wt for further investigation of this group of untreated fish scales (G1). The remaining scales were washed twice in 10%wt of NaCl solution, to remove unnecessary proteins on the surface, for 48h. Afterwards, one part of these scales was washed with distilled water forming a second group (G2), and the remaining scales were submitted to a process of demineralization and extraction of the soluble collagen, in order to achieve the third group of scales (G3). For the demineralization, it was used a solution of 0.5 mol/L of EDTA, to immerse the fish scales for 48h. Then the extraction of soluble collagen

was done by the immersion of the scales in a solution of 0.5 mol/L of Tris, HCl buffered at pH 7.5, for 48h. Scales were finally washed with distilled water and kept in it until further use.

2.2. Fish scales characterization

2.2.1. Morphology

The scales of the 3 groups were observed in the Optical Microscope (Axiotech, Zeiss) and in the SEM (Supra 40, Zeiss) in order to analyze the surface patterns.

2.2.2. Collagen Imaging

Confocal Laser Microscope (Eclipse, Ti-E, Nikon) was used to evaluate collagen architecture on different group samples, and Direct Red 80 [27] was used as unspecific stain for collagen, typically used for detection in tissue histological section. Samples were stained for 60 minutes in an aqueous solution of 0.1% of Sirius Red F3BA (Sigma Aldrich) saturated with picric acid, pH 2. After that, samples were washed for 2 minutes in 0.01M HCl, rinsed in ethanol 70%, and dehydrated with ethanol gradients, 70, 90, and 2x100%.

2.3. Fibroin films

In addition to G1, G2 and G3 groups, two more groups were created. Fibroin films with the same patterning as the scales (F1), and fibroin films without patterning (F2), in order to allow drawing conclusions about the pattern role in the cellular behavior.

The material used to mimic the fish scales patterning was fibroin. To isolate the fibroin, the process was divided in two steps: degumming and dissolution.

Degumming

Bombyx mori cocoons (kindly provided by Socio Lario, Cassina Rizzardi, Como, Italy) were degummed for 1.5 hours in a bath of boiling water containing 1.1 g/l Na_2CO_3 (10 g of silk in 1L of solution), then for 1.5 hours in another bath of boiling water containing 0.4 g/l Na_2CO_3. After the two baths cocoons were rinsed thoroughly with distilled warm water to remove salts and dried in air.

Dissolution

Fibroin-water solutions were prepared by dissolving the fibroin obtained in the previous step in 9.3 M LiBr (Fluka Chemical) aqueous solution (1g/10ml) at 65°C for 2 hours, followed by dialysis against distilled water with a 3500 Da MWCO membrane (Slyde-A-Lyzer, Pierce) in order to eliminate salts.

The concentration of the resultant fibroin solution was measured by UV-VIS spectrophotometer (Nanodrop). The solution was then diluted to a concentration of 2.5% w/v. The pH of the solution was 5.

2.3.1. Construction of fibroin films with the fish scales patterning (F1)

The first step of this process was the creation of silicon molds with the fish scales patterning. This was achieved using a plate with nine prominent cylinders with a glued fish scale with the pattern turned up on the top of each cylinder. The plate was filled with a silicon solution (Sylgard Silicone Elastomer kit 184) and the air bubbles were removed with the aid of a vacuum pump. After, the plate was transferred to an oven at 65°C for 24h. The resultant molds contained negatives of fish scales patterning.

To achieve the copy of the pattern of the fish scales, the wells containing the negatives were filled with the fibroin solution and left to dry for 3 days at room temperature. From each well resulted a fibroin film with the same pattern of the fish scales. In order to stabilize the fibroin samples, these were immersed in an aqueous solution of methanol 80% for 20 minutes, and then washed by some immersions in distilled water.

2.3.2. Construction of fibroin films without pattern (F2)

Fibroin films were prepared by casting aqueous solutions with 2.5% w/v of total protein content on polyethylene plates at room temperature for 2 days. All resulting films were stabilized using the same procedure mentioned before for the films with the scale patterning.

2.4. *In vitro* Biological tests

2.4.1. Cells culture and seeding

Before the *in vitro* tests, the scales (G1, G2, G3) and fibroin films (F1, F2) were immersed in ethanol 70% at 4°C respectively for 24 hours and 2 hours and washed with sterile distilled water.

Osteoblasts of MG63 cell line were cultured in Minimum Essential Medium (MEM) (Invitrogen), 10% Fetal Calf Serum, 1% Sodium Piruvate, 100 mM Euroclone, 2% L-glutamine 100 mM Euroclone, Non Essential Aminoacids Gibco, and 1% of Antibiotic / Antimicotic. Cells were cultured in an atmosphere of 37°C and 5% CO_2. A cell suspension with a concentration of $1*10^5$ cells/ml and 0.6mL were seeded on the samples. Cultures were maintained for 1, 3, 7 and 14 days.

The potential of fish scales to support the osteogenic differentiation of human adipose derived stem cells (hASCs) was studied. Scales from *Lates calcarifer* were submitted to a treatment for removal of unnecessary proteins on their surface by immersion in NaCl (Panreac) 10%wt during 48h, equivalent to the already mentioned for the G2 samples. All the scales were cut in circles with 10mm of diameter and immersed in ethanol 70% during 2 hours for sterilization. After this, scales were washed with sterile PBS. Cells were cultured in basal culture medium (α-MEM plus 10% FBS and 1% of A/B) and standard osteogenic medium (α-MEM plus 10% FBS and 1% of A/B, and supplemented with 10 mM beta-glycerophosphate, 50 g/mL of ascorbic acid and 10-8M of dexamethasone). hASCs at passage 2 were seeded at a density of 2000 cells/cm^2. The quantity of medium immersing each sample was of 1ml. Tissue culture polystyrene coverslips (TCPs) were used as controls. Cultures were maintained for 1, 3, 10, 21 and 30 days.

2.4.2. AlamarBlue Assay

AlamarBlue assay kit (Biosource International Inc., USA) was used to assess metabolic activity at the end of each experimental time point of the studies with MG63. The assay was performed according to the manufacturer's instructions [28].

In brief, the seeded films were incubated for 4 hours at 37°C with fresh culture medium (with reduced serum) supplemented with 10% of alamarBlue solution according to manufacturer's data sheet. A total of 3 replicates were used for each sample and each replicate

was split into 4 wells for the final reading. The references were taken from wells with unseeded samples incubated with the alamarBlue solution, wells with only culture medium, and wells only with cells. Absorbance was measured at 570 nm and 620 nm in a microplate reader (Multiskan EX, ThermoLabsystems, Finland) and the percentage of reduced alamarBlue was calculated and plotted against the time.

Statistical Analysis

Statistical Analysis was performed for the AlamarBlue assay results which are presented as means ± STDEV (Standard Deviation of the Mean). Friedman Test (non- parametric test equivalent to One Way Repeated Measurements ANOVA) was performed for each group analyzed along time. One Way ANOVA on Ranks followed by Tukey Test was performed for each time point for group comparison (SigmaStat 3.0, SPSS, Chicago, IL, USA).

2.4.3. Confocal Laser Microscope (CLM) analysis

Evaluation of cell attachment, distribution, and morphology, on fish scales (G1, G2 and G3) and fibroin films (F1 and F2) seeded with MG63, was performed by confocal laser microscopy (Nikon Eclipse, Ti-E) after Phalloidin Rhodamine (Biosource International, Invitrogen) and DAPI (Sigma Aldrich) staining according to the manufacturer's protocol. Fixation with a formaldehyde solution (4% formaldehyde in PBS solution) and permeabilization with TritonX 100 (0.2% TritonX in PBS solution) were performed before staining.

2.4.4. Scanning Electron Microscope (SEM) analysis

Morphological observations were performed with scanning electron microscopy (Supra 40 Zeiss), operating mode: high vacuum, secondary electron detector) on fish scales (G1, G2 and G3) and fibroin films (F1 and F2) seeded with MG63. At each time point, films were fixed with gluteraldehyde solution (2,5% gluteraldehyde in cacodylic buffer solution, 0,1 M) to preserve biological structures. Samples dehydration was performed by soaking in a series of aqueous ethanol solutions at increasing concentrations from 30% - 100% and drying in air at room temperature. Before SEM imaging, all samples were sputter coated with gold (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy).

2.4.5. Alkaline Phosphatase (ALP) activity quantification

Osteoblastic activity on fish scales (G1, G2 and G3) and fibroin films (F1 and F2) was monitored measuring Alkaline phosphatase activity (ALP) based on the conversion of p-nitrophenyl phosphate to p-nitrophenol.

A standard curve was firstly prepared using a kit for this assay with concentrations of pNP Standard ranging from 0 to 14 µmol/mL. The ALP assay was carried out in a 96 well-plate, where 50µl of each sample medium were put in quadruplicate, 50µl of 0,05% Triton-X (Sigma) in PBS were put in 6 wells to use as blank, and then 50µl of Buffer Solution from the assay kit were added to all. Finally the films were incubated at 37°C till the majority of the wells turned yellow, which happened after 3h20mins for the samples from days 1 and 3 of control, and after 1h42mins for the samples from days 7 and 14 of control.

2.4.6. DNA measurement

DNA content in fish scales (G1, G2 and G3) and fibroin films (F1 and F2) seeded with MG63 was measured through Picogreen, according to the manufacturer's protocol (Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). In brief, a standard curve was prepared with γDNA concentrations ranging from 0 to 2 µg/mL, and to the wells of a 96-well plate, 100µl of the standards and samples in duplicate were transferred, and 100µl of PicoGreen solution were added. Finally, the plate was read on a fluorescent plate reader, excitation of 485nm and emission 538nm.

2.4.7. Fluorescence Microscopy Analysis

Evaluation of stem cell attachment and distribution on the seeded samples was performed by fluorescence microscopy using a Zeiss Axioimager Z1 microscope (Zeiss, Germany) after Phalloidin Rhodamine (Sigma) and DAPI (Sigma) staining according to the manufacturer's protocol. Fixation with Formalin 10% (BioOptica) was performed before staining.

3 | Results and Discussion

3.1. Fish scales topography

Lates Calcarifer (white seabass) fish scales present a patterning on only one of their sides. The other side of the scales present no defined pattern, being mainly composed of minerals. Using the Optical Microscope, the patterning of the scale could be easily analyzed. The observations made in the SEM (Figure 3.1) were more detailed and confirmed the observations in the Optical Microscope.

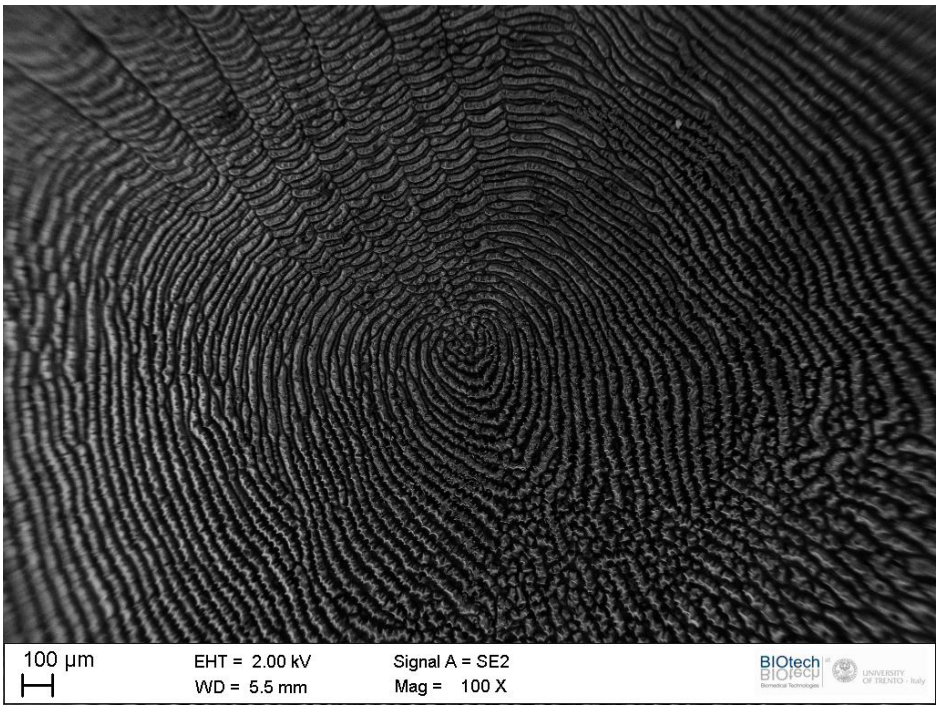


Figure 3.1 - Fish scales from group 1 observed in the SEM

The overall patterning of fish scales of white seabass is similar to a human fingerprint, being very organized and consisting mainly of parallel concentric lines. The grooves that delineate the patterning present widths of about 10-20 µm. Many scales of the three groups of scales created (G1, G2, G3) were observed and all of them presented similar patterning, although the third group had a less incisive pattern, most probably due to the performed treatment that removed soluble collagen.

The Confocal Microscope analysis using a red staining to identify the collagen confirmed that the scales are rich in collagen, presenting a more intense staining in the patterning lines (Figure 3.2), which is due to a higher concentration of collagen in those areas.

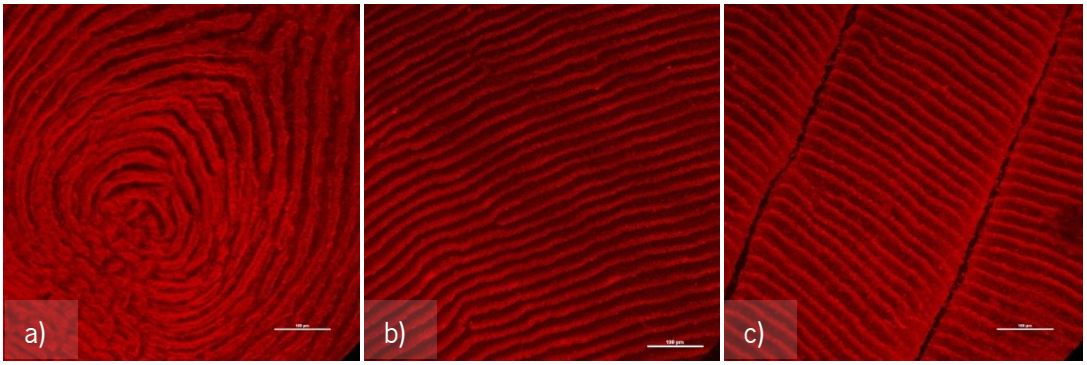


Figure 3.2 - Fish scale observed in the Confocal Microscope with Direct Red 80 staining for collagen identification: a) center, b) side and c) top areas of the scale

Fish scales presented an attractive structure for cell culture due to presenting a organized and regular patterning and also being highly composed of collagen which is a major protein in the human body.

3.2. Osteoblast Behavior

Theoretically, the percentage of reduced Alamar Blue should be continuously upward with the advance of the days in the case of cellular proliferation, what would mean an increasing of the cells number. In the graphic of Figure 3.3 it is shown the percentage of reduced Alamar Blue based in the calculations from the absorbance results.

Besides the AlamarBlue test, cell proliferation on the different films was evaluated through the Picogreen assay. The results of this test confirmed the main ideas collected from the AlamarBlue test, being the same overall tendencies shown, although was evidence cell proliferation for all groups of samples.

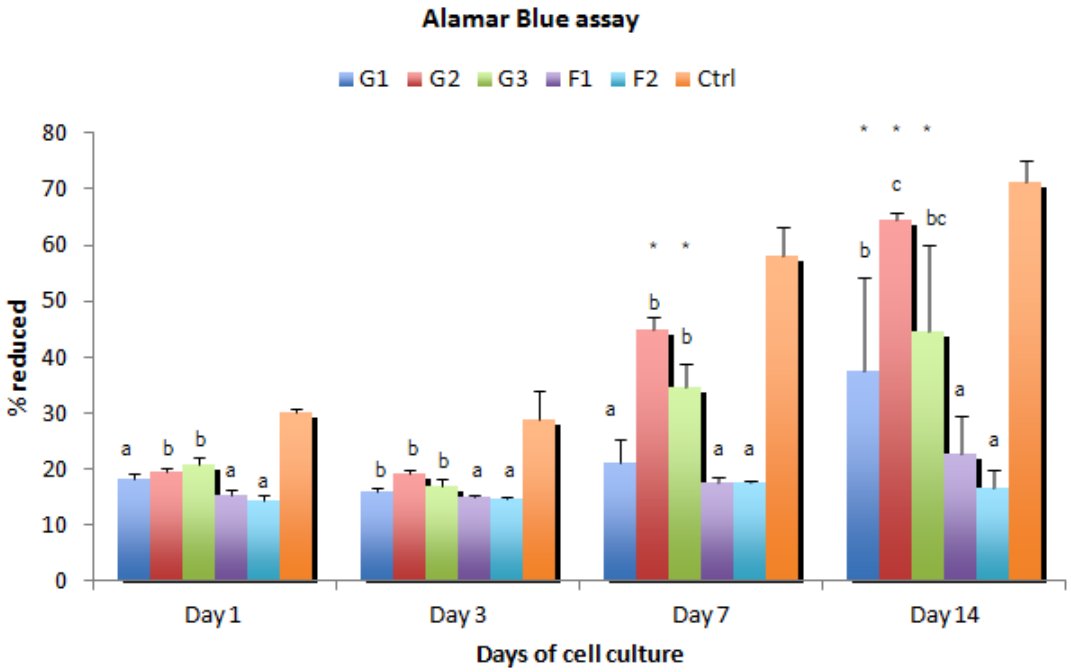


Figure 3.3 - Percentage of reduced Alamar Blue for each group in the different days of control. Values are mean ± STDEV, bars with like characters are not significantly different (P>0.05). Abbreviations: G1 – untreated fish scales; G2 – fish scales after a treatment for unnecessary proteins removal from the surface; G3 – fish scales after demineralization and collagen extraction; F1 – fibroin films with the fish scales surface pattern; F2 – fibroin films without pattern; Ctrl – controls with cells seeded in the wells-plate.

Analyzing the resultant graphic (Figure 3) is perceptive the tendency of the three groups of fish scales (G1, G2 and G3) to have a growth in the percentage of reduced AlamarBlue along the 14 days of culture, that means an increase of cells amount. After 7 days of culture, the cells amount presented already a significant increase for G2 and G3 of fish scales. The most significant cellular proliferation along the 14 days was for group 2 (fish scales after a treatment to remove unnecessary proteins from their surface).

Concerning the groups of fibroin, the percentage of reduced AlamarBlue did not present a significant growth in the 14 days. In spite of that, DNA measurement results showed osteoblasts proliferation for the groups of fibroin, which means that osteoblasts exhibited less metabolic activity on these samples.

Therefore the fish scales provided good conditions for cellular proliferation, such as good chemical composition that allowed cellular attachment and induction for multiplication.

The ALP test measures the activity of alkaline phosphatase by the quantification of hydrolyzed pNPP in the samples, being an indicative of the cells ability to mineralize.

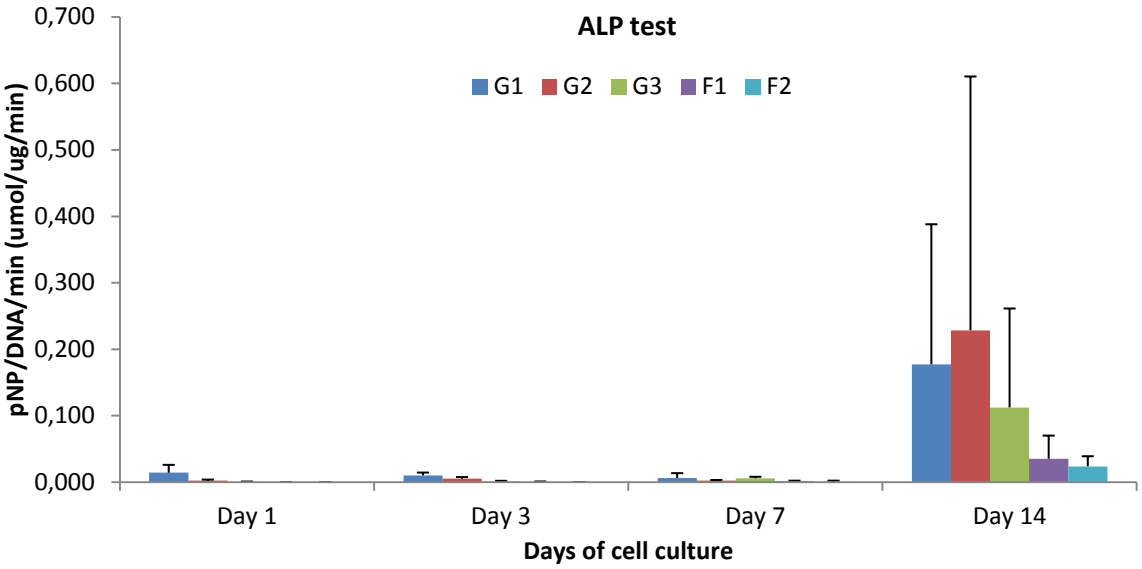


Figure 3.4 - Quantity of pNP enzyme produced per DNA µg per minute for each group of samples on different days of control. Abbreviations: G1 – untreated fish scales; G2 – fish scales after a treatment for unnecessary proteins removal from the surface; G3 – fish scales after demineralization and collagen extraction; F1 – fibroin films with the fish scales surface pattern; F2 – fibroin films without pattern.

By the Figure 3.4 analysis it can be deduced that the ALP activity on the first 7 days after the seeding is practically null, however the samples of day 14 already started showing some activity. The samples of group 2 are the ones presenting higher ALP activity showing more potential for the mineralization induction.

The fibroin samples presented very low activity, although Altman G. et al. [29] showed that fibroin films induced osteoblasts mineralization *in vitro*, when the films were chemically modified with the peptide RGD to promote integrin interactions for adhesion.

The samples analyzed by Confocal Microscope were stained with Phalloidin Rhodamine to identify actin filaments in the cytoplasm, and DAPI to identify the cells nuclei.

In Figure 3.5 it is possible to see the cell behavior in the different samples along the the time, with the exception of the samples F2 for reasons that will be explained further ahead. On the first days many cells were already noticed, being visible their alignment along the axis of the pattern of the scale and that they acquired an elongated shape. With the advance of the days it could be noticed more zones with cells and an increase of cellular density. Even in some situations when there was high cell density and existed some superimposition between the cells the alignment and elongated shape was maintained. This was the cell behavior for all the types of samples, although F1 presented fewer cells with a less elongated shape than the cells in the scales, but still following the pattern in the fibroin.

Walboomers et al. [30] studied the behavior of fibroblasts in micro-grooved polystyrene and concluded that the width of the grooves that form a pattern has high influence in the cells behavior: for a cell to sense the topography of the surface on which it is seeded, the physical cues should not be greater than the actual size of that cell, although the grooves have to be wide enough to allow cells to descend. Ber et al. [13] also showed that the cells behavior was not affected when the grooves were wider than the cells size. The osteoblasts culture in the scales micro-pattern seems to be ideal, since the normal size of osteoblasts is about 20-30μm in diameter and the scales grooves are of 10-20μm, allowing the cells to descend but also narrow enough for cell shape and alignment.

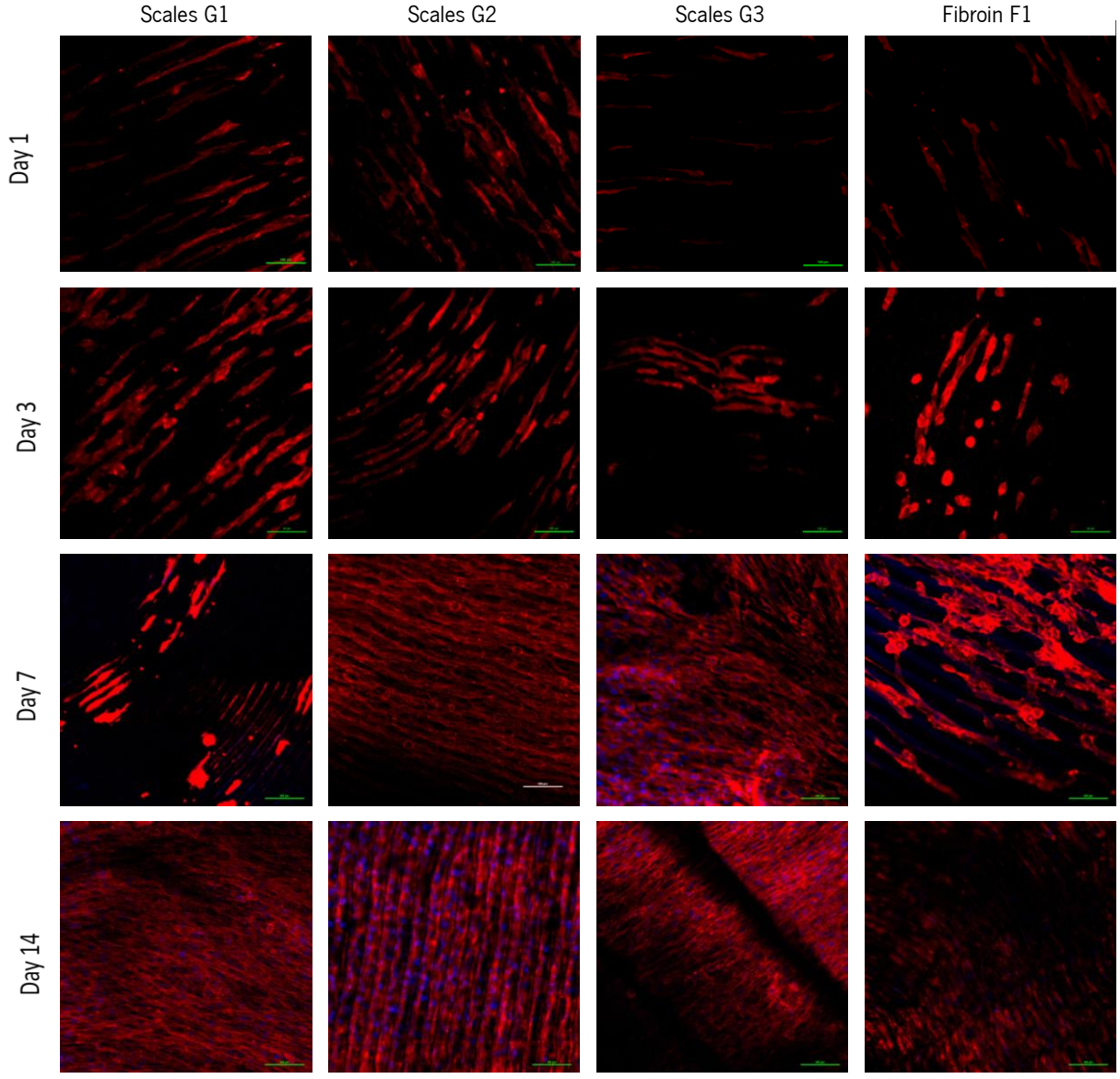


Figure 3.5 - Results of the Confocal Microscope observation after stainings with DAPI and Phalloidin Rhodamine. The subscripts indicate the type of sample and the time period after the cellular seeding. The scale bar is 100 μm.

The samples of fibroin without any pattern (F2) had some zones with cells, but those presented different shapes between them. Along time the cell behavior did not change but could be observed a raise on cellular density.

The observations in the SEM (Figure 3.6) confirmed the results of the Confocal Microscopy. The cellular orientation due to the existent pattern and their elongated shape was perceptible.

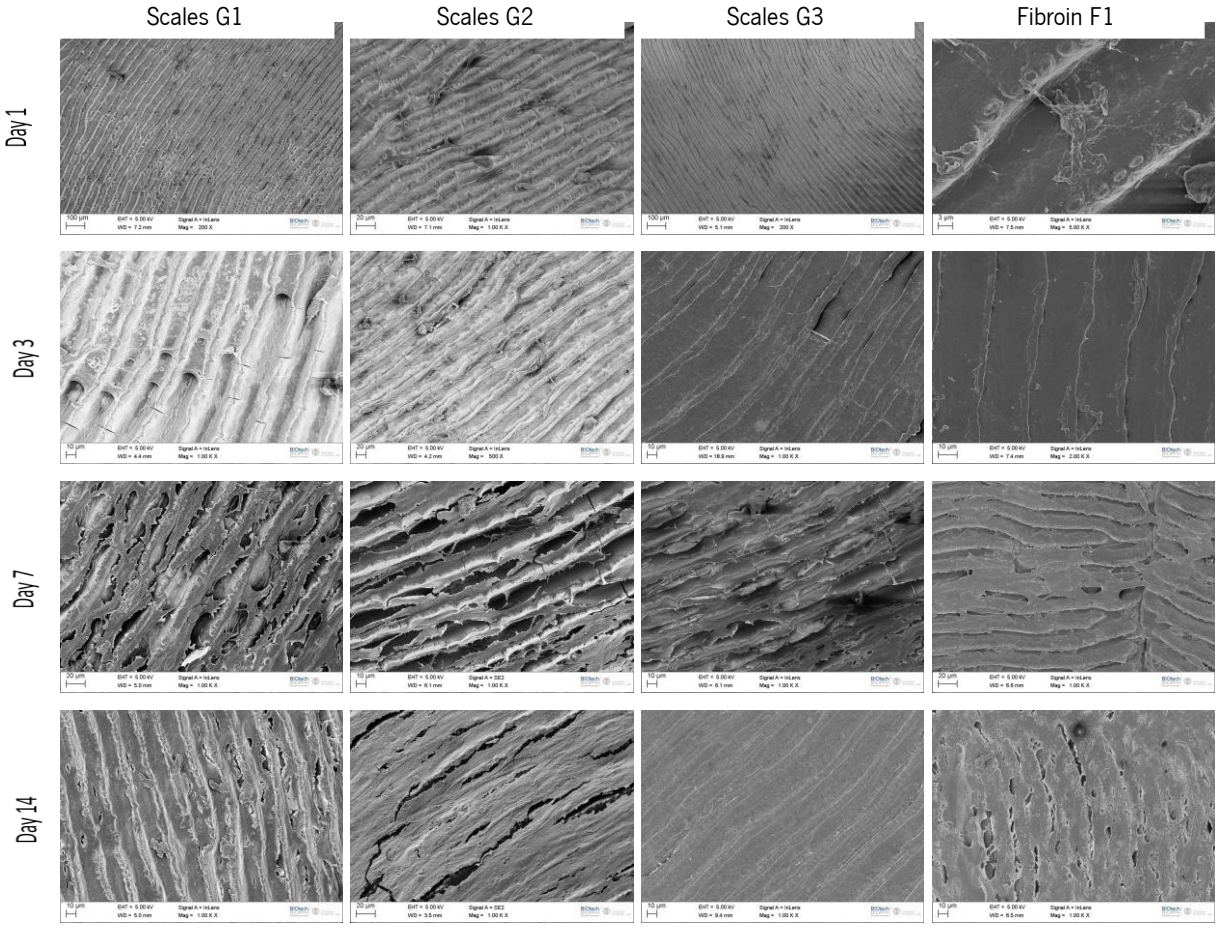


Figure 3.6 - Results of the SEM observation. The subscripts indicate the type of sample and the time period after the cell seeding.

The fish scales samples of the first time point day presented quite similar results between each other, having some zones with cells although difficult to identify due to being between the scales pattern. For longer time points an increase of cell density for every sample was observed. The samples of day 14 presented already high density of cells as can be observed for example in *G2 day 14* corresponding image, where the cells even overlap the pattern.

The F2 samples presented different shapes and were located and oriented uncritically. Cell spreading was not influenced by any constrictions making them to acquire variable shapes and orientations.

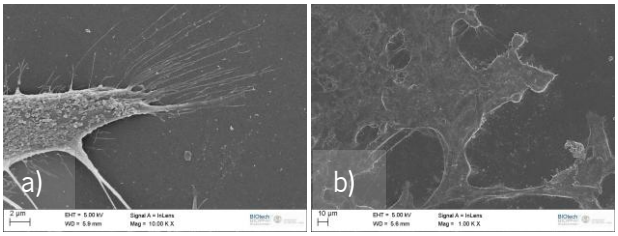
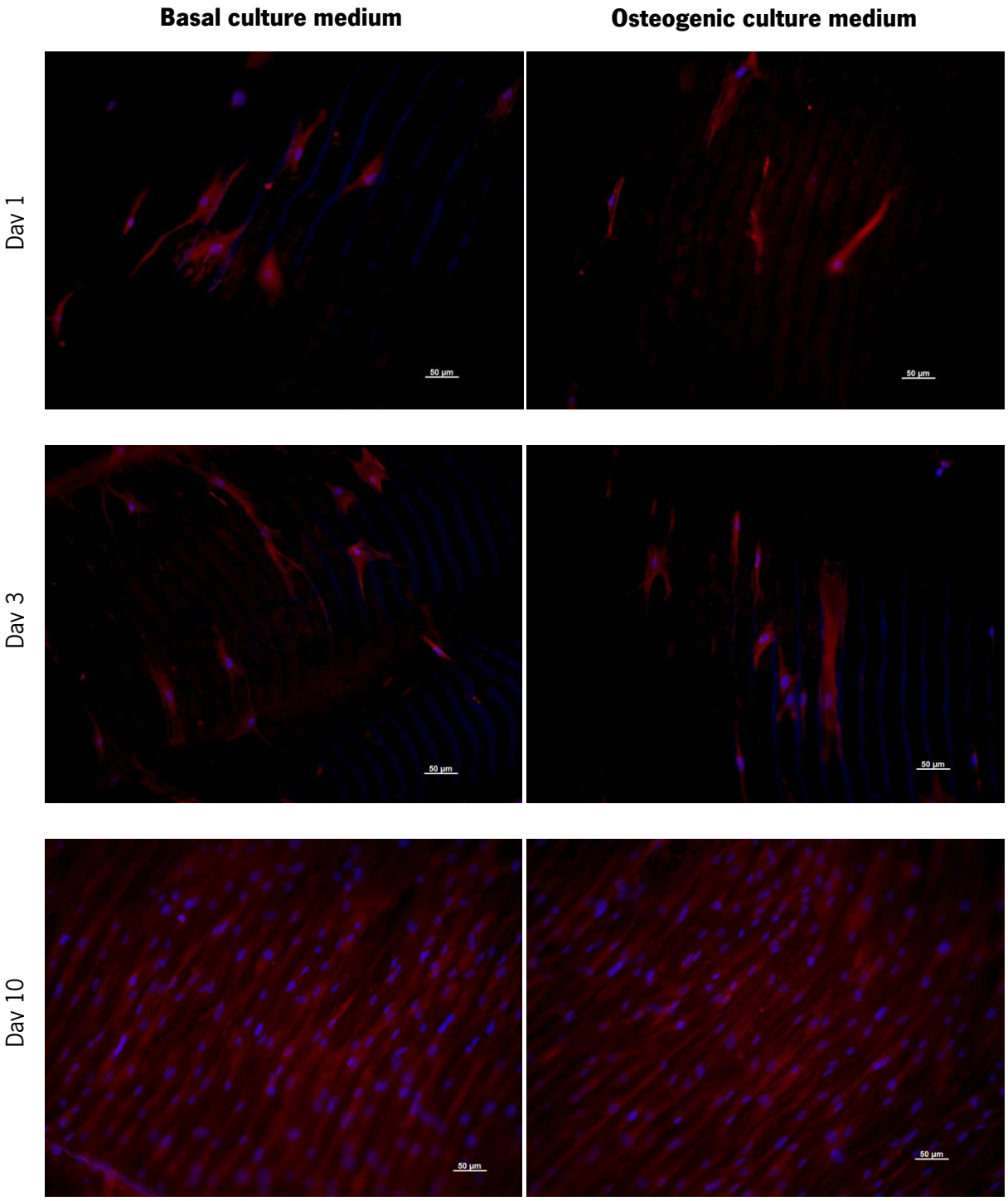


Figure 3.7 – Samples of F2 after: a) 1 day; b) 7 days. Along time cells spread without any specific orientation.

3.3. Stem cells (hASCs) Behavior

The study with hASCs in fish scales by Fluorescence Microscopy permitted to observe that the stem cells when seeded were able to descend into the scales pattern microgrooves. In Figure 3.8 we can observe a sequence of images showing the cell behavior along the 30 days of culture for the fish scales when submitted to a basal culture medium and a osteogenic medium.

From the analysis in the Fluorescence Microscope no significant difference between the samples submitted to basal culture medium and osteogenic culture medium could be detected. Along the days was obvious the growth of cellular density in the samples. In the first 3 days after the cells seeding was already perceptible the cells spreading and acquisition of a narrow and elongated shape. After 10 days the cellular density was already high but the cells still presented an orientation according to the scales pattern axis. At day 21 there were already many zones with cellular superposition that led to a loss of the cells orientation, in spite of in some zones this alignment with the pattern and also the elongated form of the cells was still visible. After 30 days cells were already forming several layers that induced the cells to be oriented randomly and without morphologic criteria.


Basal culture medium
Osteogenic culture medium
Day 1
Dav 3
Day 10
50 µm

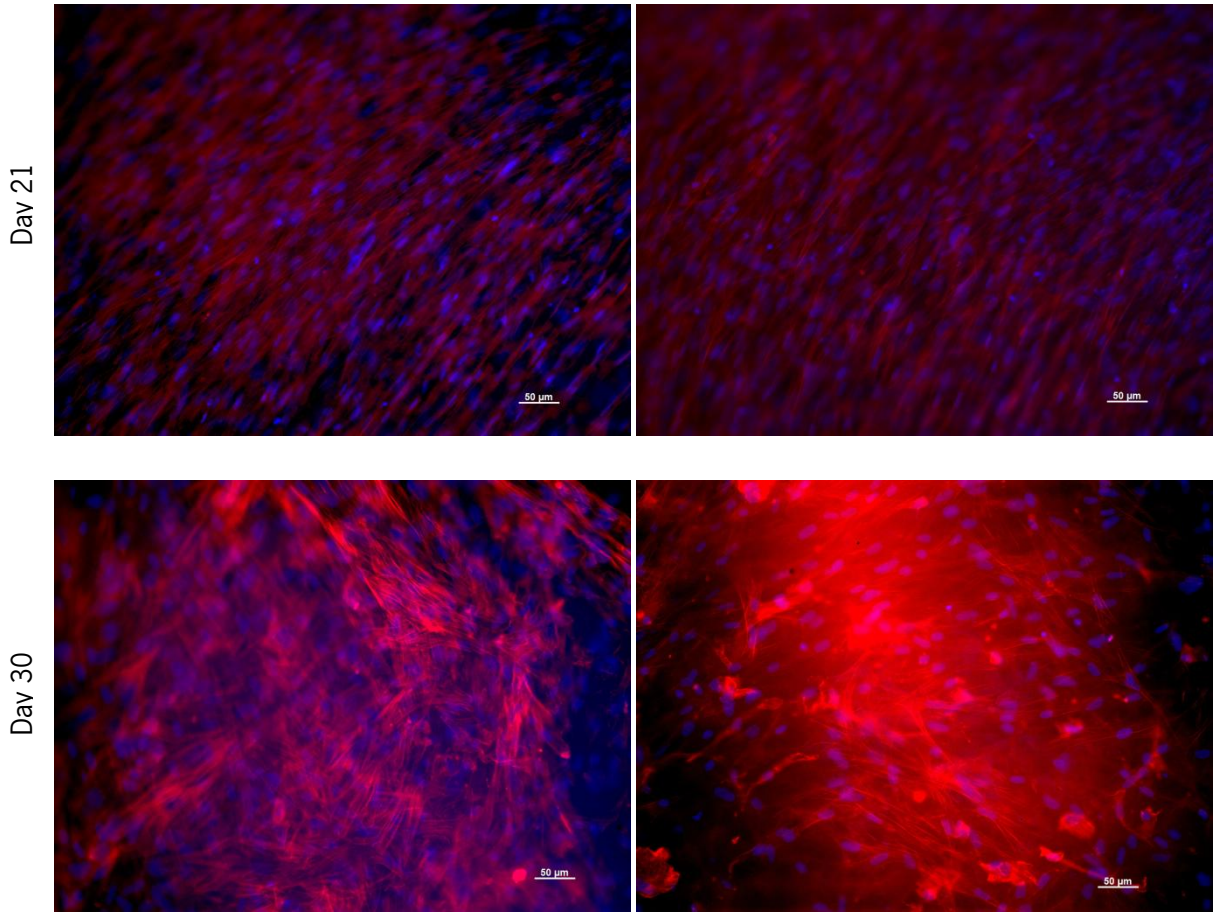


Figure 3.8 - Results of the observation in a Reflected/Transmitted Light Microscope after stainings with DAPI and Phalloidin Rhodamine. The subscripts indicate the type of culture medium used and the time period after the cellular seeding. The scale bar is 50 µm.

4 | Conclusion

The fish scales of *Lates Calcarifer* presented a very organized pattern consisting mainly of parallel concentric lines. The three groups of scales created could support a considerable cellular proliferation showing good capacity for cell attachment, especially G2 (fish scales after a treatment for removal of unnecessary proteins from the surface). The microgrooves existent in the scales create a pattern, which influences cell spreading and cause the cells to be narrower and aligned with surface microgrooves.

It may be true that bone regeneration can be already achieved without the aid of patterns, nevertheless there is always room for improvement. Patterns could induce faster bone formation by shortening the cells reorganization periods.

Our results suggest that the fish scales composition and topography are responsible for a good cell proliferation and induce modifications in cell organization and morphology. Further studies will be needed to conclude about the hASCs osteogenic activity when submitted to the fish scales pattern.

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CHAPTER IV.

GENERAL DISCUSSION AND FUTURE WORK

General Discussion and Future Work

Scales of *Lates calcarifer*, also known as White Seabass, were collected in a market in Thailand and were not submitted to any treatment till the start of this study. They were then divided in three groups: untreated fish scales (G1); scales after a treatment for removal of unnecessary proteins from the surface (G2); and a third group of scales after demineralization and extraction of the soluble collagen (G3). The pattern of these scales was analyzed and the 3 groups presented a similar organized pattern consisting mainly of parallel concentric lines and highly composed of collagen, being the pattern in G3 less incisive due to the soluble collagen extraction.

The potential of the 3 groups of scales created as scaffolds for cell proliferation and the behavior of osteoblasts MG63 were evaluated. To draw conclusions about it, fibroin films with the same pattern of the scales and without any pattern were also created. The scales showed the ability to support cell proliferation obtaining better results than the fibroin samples, both with and without pattern, being this mostly due to their composition of hydroxyapatite and collagen [1] [2]. The scales of G2 (without undesirable proteins on the surface but still with the presence of high quantity of collagen) were the ones that supported the highest cell proliferation. The ALP test results indicated that osteoblasts on the scales presented some activity after 14 days of cell culture, although this activity was practically inexistent for the fibroin films. The scales of G2 were again the ones presenting more promising results. Analysis of the samples in the Confocal Microscope and SEM were conclusive about the osteoblasts behavior on the fish scales. The cells presented an orientation according to the scales pattern alignment, acquired an elongated and strait shape. Then, the mechanical stress [3] granted by the scales pattern played a determinant role in the osteoblasts shape and in the way of their distribution, being this phenomenon denominated as contact guidance [4].

The culture of stem cells (hASCs) on fish scales presented accordant results with the previously tested osteoblasts behavior. The scales pattern still guided, oriented and led the cells to acquire a narrow and elongated shape at least till the cells superposition surpassed the grooves of the pattern. The stem cells behavior was similar when the samples were submitted both to a basal culture medium and osteogenic culture medium.

Tissue regeneration is a three dimensional event and in this context the presence of patterns in biomaterials could be helpful with the cells guidance. Then, scales pattern presence could shorten the tissue formation and the cells reorganization period.

In this work, also collagen films with a pattern similar to the fish scales were achieved. Although the cross-linking method used using Genipin was not enough to guise the collagen hydrophilic nature that led to films deterioration via hydrolysis after some time immersed in culture medium for example. This would also make it difficult to use in long-term applications. Ber et al. [5] tried to overcome this difficulty with the collagen films subjecting these to a variety of stabilization treatments. They observed that calcium phosphate deposition was a very good treatment method, so in future works the collagen films should be submitted to this treatment to acquire a greater capability to resist in the presence of water. Curiously the films would also get a similar arrangement to the fish scales, being composed of collagen and calcium phosphate.

Fish scales pattern showed great potential both for osteoblasts MG63 and stem cells (hASCs), although further studies should be performed to conclude about the hASCs osteogenic activity when submitted to the fish scales pattern. For future work is believed that this pattern or adaptations of this depending on the purpose should be tested.

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APPENDIX

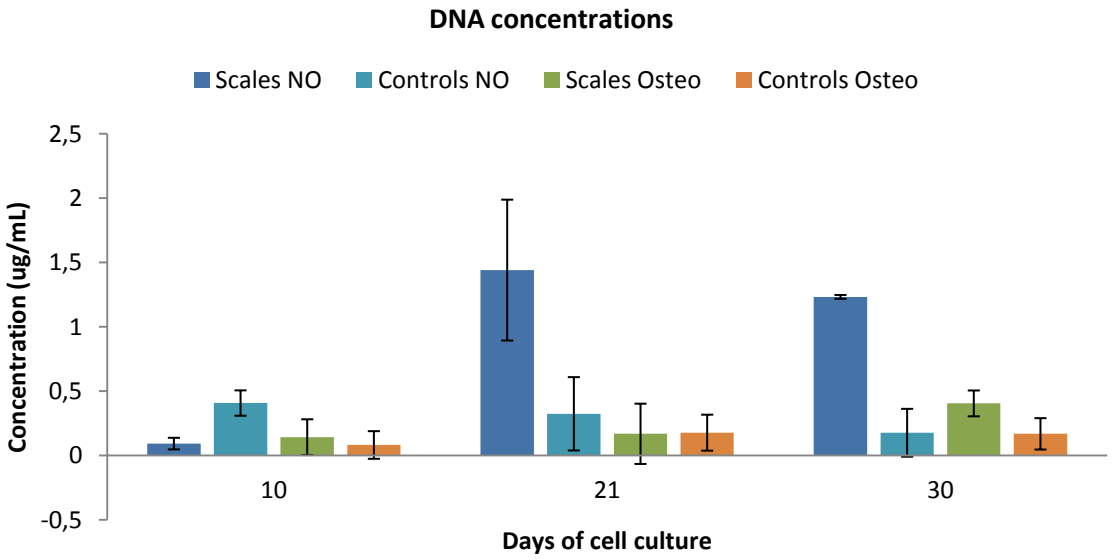
Collagen films

In this work, collagen films with a pattern similar to the fish scales were achieved with the procedure described in the *Materials and Methods* section of this work. Although the cross-linking method used using Genipin was not enough to guise the collagen hydrophilic nature that led to films deterioration via hydrolysis after some time immersed in culture medium for example.

The collagen films lack of stability in culture medium was an important factor that did not allow results of these films with stem cells.

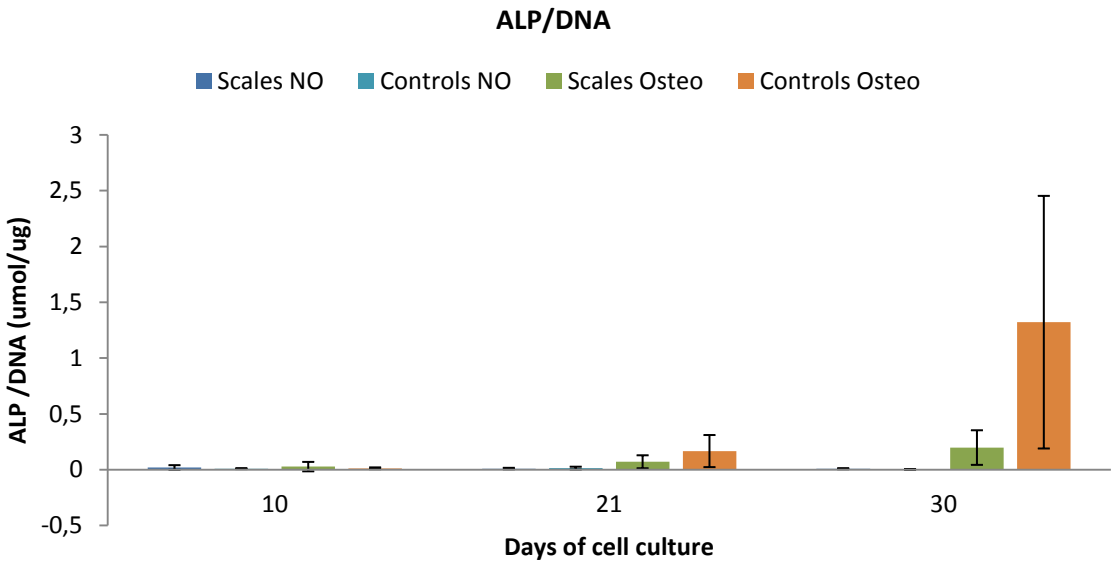
DNA measurement and ALP test – stem cells

The results obtained for DNA and ALP were not concordant to the expected and to the previous observations regarding both fish scales and controls.



By the previous graphic analysis is easily perceptible these results are not according to the expectable since the controls do not present an increase of DNA concentration along the days, being this evidential that there was not cell proliferation. This strange behavior was indicative that these results could not be acceptable, since the normal behavior would be the cell proliferation in the controls along the days. Besides not being a quantitative and conclusive measure, in the observations in the microscope of both fish scales and controls could be

perceptible cell growth and the existence of higher cell density along the days that contradict these results.

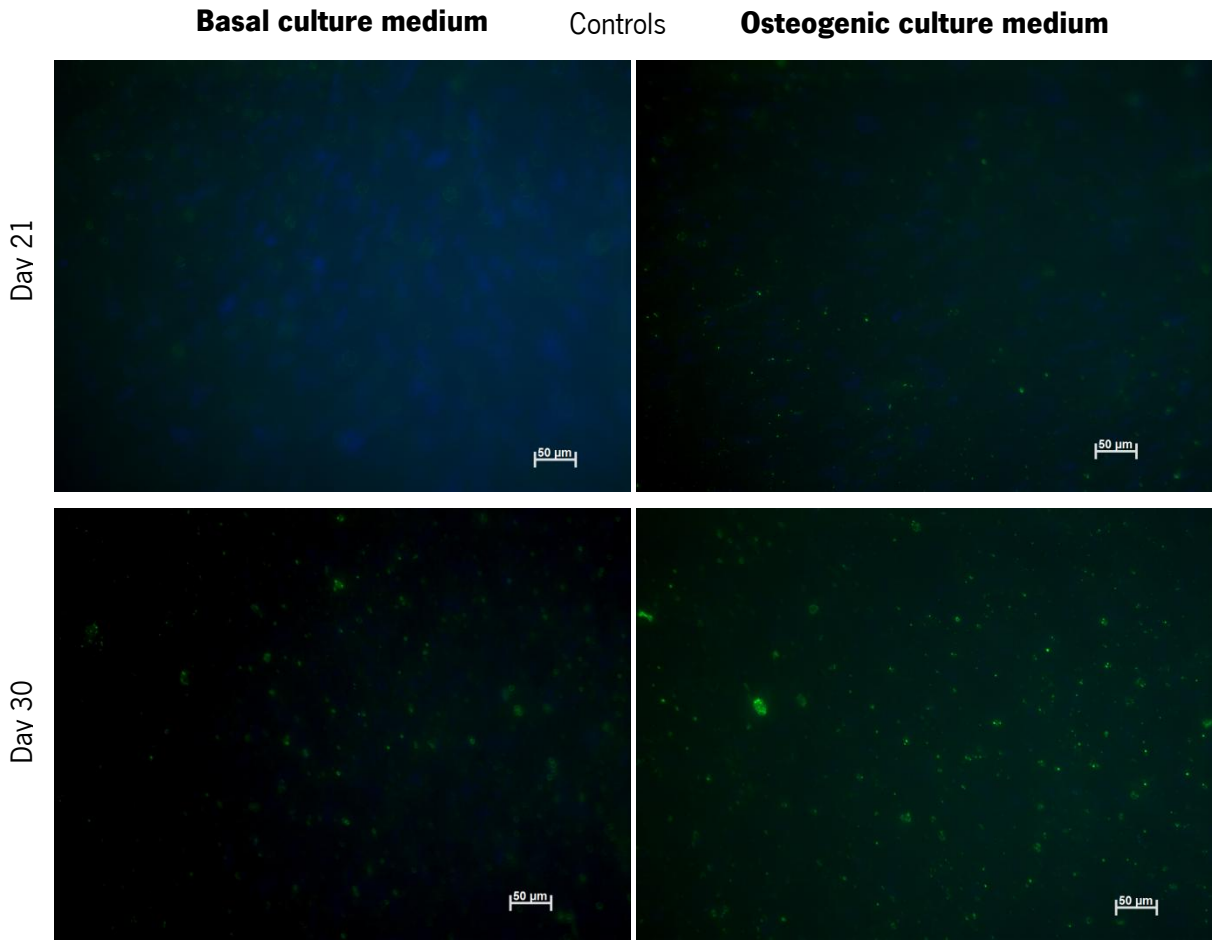


Since the ALP test was performed over the same samples of the DNA test, and the results are also directly connected to the DNA results, no assertive conclusions could be taken about the cellular activity.

Immunocytochemistry test – stem cells

This test was performed to identify the presence of osteocalcin that would be an indicative of cell mineralization since it is a secreted protein associated with the mineralized matrix of bone.

The results obtained of this analysis were not clear since fish scales presented auto-fluorescence that can be due to their highly mineralized structure. This did not allow clear and perceptible results regarding the fish scales. Nevertheless with the controls consisting of coverslips was already possible a better observation of the presence of osteocalcin since these did not present a background prejudicing the observation. The images got for these controls are nextly presented.



For the controls the presence of osteocalcin was more evident only after 30 days of culture and for the osteoinductive culture medium.