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Enhancing Collagen Functionality with Bio-Stimulating Peptide Derivatization

Student: Mario Grossi
ID number: 1108655

Thesis Supervisor:
Prof. Lorenzo Scalise

Co-supervisor:
Dr. Paolo Fattori

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Ai miei genitori,
che hanno reso possibile tutto ciò

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Abstract

Regenerative Medicine and Tissue Engineering are advanced fields of research dedicated to the development of novel approaches for the repair and regeneration of damaged or diseased tissues. One of the most promising strategies involves the use of natural biomaterials that mimic the properties of biological tissues, often modified to enhance their intrinsic characteristics. This study is part of a broader project focused on enhancing collagen scaffolds with specific signaling molecules to achieve targeted cell programming and direct desired actions within tissues and cells.

In this pilot study, the goal is to improve collagen scaffold functionality through decoration with RGD, a bioactive peptide known for its ability to promote cell adhesion and osteogenicity.
To achieve the binding between collagen and RGD, EDC was used as crosslinking agent following three different procedures:

- **P1**: Collagen, EDC 1 mM, RGD 1 mM
- **P2**: Collagen, MES 20 mM, EDC 1 M, RGD 1 mM
- **P3**: Collagen, Acetic Acid 10 mM, MES 10 mM, EDC 0,5 M, RGD 0,5 mM

The bond formation was verified using several analytical techniques, including UV-VIS spectrophotometry, fluorimetry, and high-performance liquid chromatography (HPLC). Additionally, in vitro cellular assays with mesenchymal stem cells (MSCs) were conducted to evaluate the cytotoxicity, cell adhesion, and potential enhanced osteogenicity of RGD-decorated collagen.

The results obtained from UV-VIS spectrophotometry, fluorimetry, and HPLC confirm that the collagen scaffold was successfully decorated with RGD using all three procedures, with the highest reaction yields obtained using P2 and P3.
According to in vitro tests, scaffolds decorated following P2 and P3 were found to be cytotoxic, while the enhanced scaffold produced with P1 promotes cell adhesion, cell proliferation, and improves the osteogenicity of the collagen scaffold. Indeed, the P1 RGD-decorated scaffold exhibited ALP activity (an indicator of osteogenicity) that was 46.7% higher compared to undecorated collagen in standard DMEM media and 81.5% higher in osteogenic media.

Contents

List of Figures

List of Tables

Chapter 1

INTRODUCTION

1.1 REGENERATIVE MEDICINE

Regenerative Medicine (RM) is an interdisciplinary and extremely complex field focused on the development of new therapies capable of rejuvenating, repairing and replacing damaged body parts.
Its main purpose is not the treatment of the symptoms but the functional restorement of tissues and organs within the human body, since it, unlike some lower vertebrates, is not capable of self-regeneration [1,2].
Over the years, RM has developed numerous solutions, focusing on different aspects and progressing hand in hand with the technology. Nowadays, RM is an extremely interdisciplinary field. It combines knowledge from tissue engineering, cell biology, materials science, life sciences, stem cell biology, gene therapy, etc.
Considering modern medicine, 1954 was a milestone for RM in that the first successful transplantation of an organ, a kidney, occurred. Since then, numerous transplants of different types (autologous, allogeneic, or xenogeneic) have been developed, becoming a common and successful practice. Nevertheless, numerous challenges and issues are still associated with this practice, such as a lack of donors (compared to demand) and the rejection of the transplant. To overcome the latter, over the years several drugs such as immunosuppressants have been developed, but these can cause side effects such as infections, increased risk of certain cancers, and other health problems [3].

Nowadays, RM is therefore trying to overcome these obstacles by developing new approaches for tissue and organ regeneration and healing, such as tissue engineering and cell/gene therapy. Currently, most studies focus on in vitro organ creations, 3D printing of tissues, and methodologies capable of inducing tissue regeneration directly in the human body. These practices include the use of specialized cells and genetic manipulation, with the ultimate goal of creating artificial

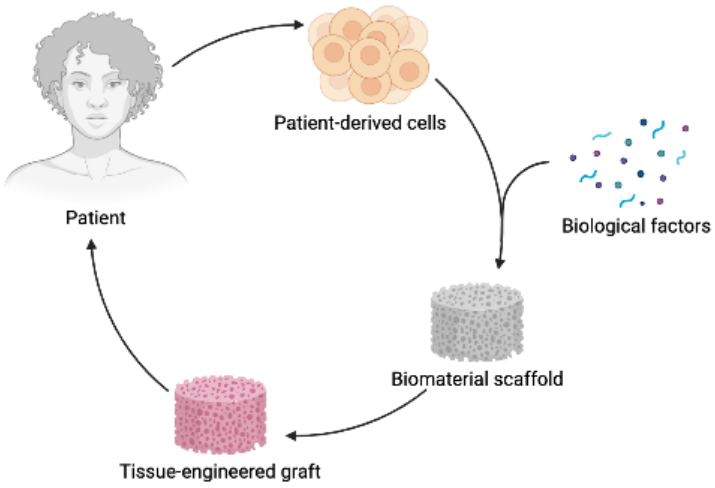
functional organs and tissues [4].

Currently, RM focuses on two main fields: the first and most significant is cellular and acellular tissue engineering, while the second is composed of cell and gene therapy. In the following paragraphs, each of them will be analyzed in detail.

1.1.1 Tissue Engineering

Tissue Engineering (TE) is an extremely relevant field of RM. In fact, nowadays, it's possible to indistinctly talk about of TERM (fusion of TE with RM) [5].
TE aims to create artificial tissues and organs that can replace, repair or improve damaged or diseased tissues in the human body. It thus combines principles of engineering, biology, chemistry and medicine.
Based on the used elements, TE can be divided into Cellular TE (CTE) and Acellular TE (ATE). As reported in Fig. 1.1, the basis of CTE and all its applications is the use of three fundamental and distinct elements: cells, scaffolds, and biomolecules. These components are combined together to promote in vitro or in vivo tissue growth.

Figure 1.1: The three fundamental elements of CTE [F1]

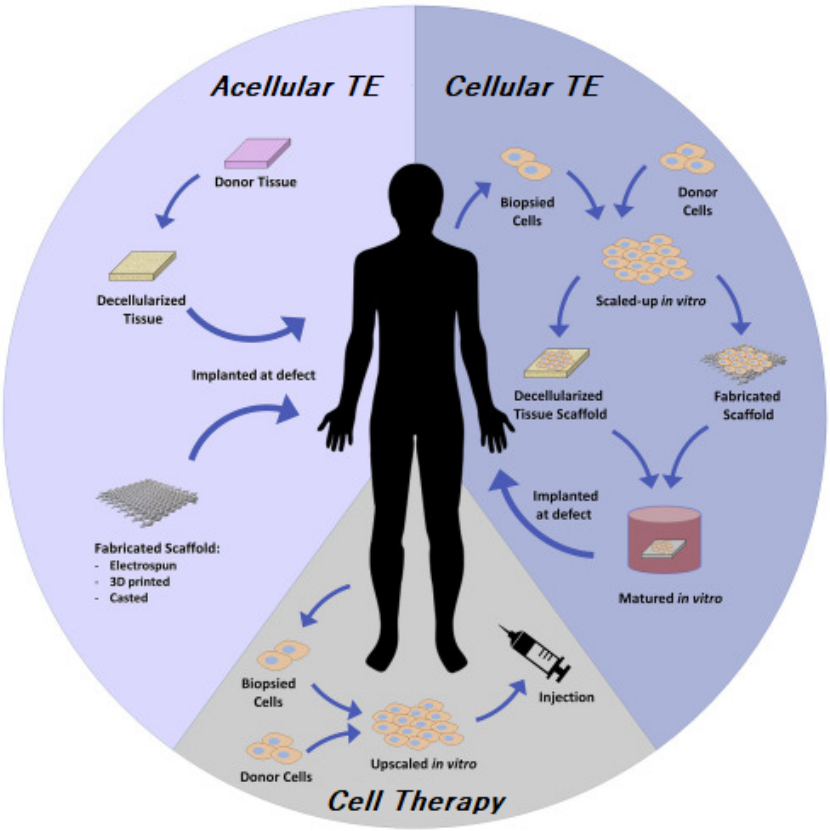


In the case of ATE, scaffolds are used as a single element. They are designed to provide a three-dimensional environment able to support tissue growth and regeneration without cell addition. The purpose is to create an environment that promotes the migration, proliferation, and differentiation of endogenous cells present in the surrounding tissue, rather than providing cells directly through transplantation.
In some applications, it's also possible to use cells as single element: it's the case of Cell Therapy

(CT). It consists in the use of cells as therapeutic agents to treat diseases or conditions. This branch of RM will be analysed separately, as it is not directly included in the TE field.

The main differences among the three techniques are reported in Fig. 1.2.

Figure 1.2: Cellular TE, Acellular TE and Cell Therapy [F2]



In the section below will be separately analyzed the three fundamental elements of CTE.

CELLS

Cells play a key role in TERM, as they possess an extremely high therapeutic potential that is difficult to replace through other chemicals or drugs. In recent decades, cell-based therapy has attracted considerable attention, and the global market for cell-based treatments is projected to grow rapidly, with a projected compound annual growth rate of 14.9% from 2017, reaching 7.24 billion by 2025 [6].
From the dawn of CTE reaching the present day, two types of cells have mainly been used: mature cells and stem cells. Due to their complete differentiation, mature cells have some limitations, mainly related to sampling and proliferation. This is precisely why stem cells are currently the

most studied and used cells, as, due to their regenerative capacity and versatility, they offer a more promising approach for TE and RM [7].
Currently, two different types of stem cells are used in preclinical and clinical studies:

1. **First-generation stem cells:** they are multipotent and include hematopoietic stem cells (HSCs), mesenchymal/stromal stem cells (MSCs), and stem cells derived from fetal tissue. MSCs can be harvested from various tissues, including bone marrow, adipose tissue, and umbilical cord [8], thus they are the most widely used. They exhibit a fibroblastic appearance, express specific surface markers, possess the ability to self-renew, and can differentiate into mesodermal lineages [9]. A special subcategory of MSCs is those derived from the umbilical cord (uMSCs), which are characterized by high proliferative and differentiative capacity and low immunogenicity.

2. **Second-generation stem cells:** they are pluripotent and include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs, obtained from the inner cell mass of blastocysts, can differentiate into various cell types in specific cultures. Although differentiated cells derived from ESCs have been used in clinical trials, their clinical application has been limited because of possible immune rejection, probable teratoma formation, and also due to ethical issues [10].

Recently, next-generation stem cells have been introduced to expand the clinical applications of stem cells. These genetically modified stem cells benefit from improved functionality, specificity, and responsiveness compared to their original counterparts and could be used in personalized medicine [7]. These types of stem cells can be generated by genetic modification.

Currently, MSCs are the most widely used cells in CTE, as they have shown significant potential in various therapeutic applications. However, it is also important to point out the limitations, such as harvesting and the need for rigorous donor selection, as age and health status can greatly affect the quality and function of MSCs [11].

SCAFFOLD

The use of scaffolds in TE was introduced to overcome the many disadvantages of direct cell suspensions, which had a very low cell survival rate once administered. Some research has shown that only about 5% of injected cells remain at the injection site on the first day after transplantation, suggesting a survival rate potentially lower than 1% [12]. In contrast, using scaffolds makes it possible to produce three-dimensional tissue systems and implement effective cell delivery strategies.
Scaffolds can also be used alone, in order to promote the proliferation, adhesion and migration of endogenous cells: it's the case of ATE.

Different types of scaffolds are distinguished according to their applications, structure, composition, and fabrication technique. The design and fabrication of scaffolds represent an extremely complex procedure, that requires specific properties followed by the validation of numerous quality control points. Such processes require in-depth knowledge of tissue structure and metabolism. The ideal scaffold must possess key characteristics such as biocompatibility, specific biodegradation rate, adequate mechanical properties and proper architecture.
In the following part will be listed the main features to be considered during the design and fabrication of a scaffold for CTE or ATE applications.

Biocompatibility: it certainly represents one of the fundamental and mandatory characteristics of a scaffold and is closely related to the type of material used. This property can be described as a combination of biostability, biofunctionality and bioactivity [13]. A biocompatible material is accepted by the host organ or tissue without triggering chronic inflammation, inappropriate immune reactions or allergic responses. It's fundamental that the biomaterial mustn't be carcinogenic, mutagenic, teratogenic, or toxic. However, it should be emphasized that biocompatibility is also influenced by the type of application. Phenomena such as the interaction of the biomaterial with the surrounding environment, residence time in the body, surface properties (physicochemical) and mechanical properties of the biomaterial can influence its biocompatibility.

Materials: all the materials used to create a scaffold in CTE or ATE must be biocompatible, and thus are called biomaterials. Some of the most widely used include:

- Natural polymers: known for their broad biocompatibility, natural polymers share many characteristics with body tissues and organs, being obtained from natural resources. They also bear some similarity to the extracellular matrix (ECM) [14], preventing immune responses. However, natural polymers often have insufficient mechanical properties and a high degradation rate. This limits their applicability.

- Synthetic polymers: are characterized by adequate mechanical properties and a modulable rate of biodegradation. Synthetic polymers are designed for specific applications and can be modulated in composition, resulting in final scaffolds with desired mechanical properties and degradation times. However, they have an increased risk of inducing immune reactions and chronic inflammation or not being easily incorporated into host tissue [7].

- Inorganic biomaterials: include metallic materials (such as titanium and its alloys) and ceramic materials (such as calcium phosphates and silicates) and are mainly used in dental and musculoskeletal applications [15]. These biomaterials boast cellular compatibility, adequate mechanical properties, and osteoconductivity [7]. Based on their interaction with the host tissue, inorganic biomaterials are generally classified into three groups: bioinert, bioactive, and bioresorbable. The first group refers to biomaterials that do not interact with the surrounding tissue (such as alumina, zirconia, and titanium) and usually create

a layer of fibrous tissue at the interface. Bioactive materials, (such as bioactive glass and calcium phosphates) can interact positively with body tissues and thus are easily incorporated into the host. Resorbable biomaterials, instead, are digested by the body within the exact time required for the tissue healing process, without leaving any adverse effects [7].

An extremely used material in CTE and ATE is the ECM [16]. It can be considered a particular type of natural polymer. In fact, originating from a natural microenvironment, the ECM contains numerous vital macromolecules, including fibrous proteins such as collagen and elastin, glycoproteins such as laminin and fibronectin, and proteoglycans [17]. These components are joined together to form the structure of the tissue.
In addition, ECM components, secreted by the resident cells of each tissue, can regulate cell behaviour and function, influencing vital processes such as attachment, proliferation, migration, differentiation, and cell death [18]. To prepare natural ECM, organ and tissue decellularization procedures have been developed, employing mechanical, chemical, enzymatic or combined procedures [19]. ECMs have been successfully used in the fabrication of scaffolds in TE. This material promotes the bioactivity of constructs and induces cellular responses that promote more efficient and specific tissue regeneration.

Fabrication techniques: there are two main scaffold fabrication techniques:

- Traditional prototyping: includes all the methods widely used before the introduction of 3D printing in TERM. Several methodologies have been adopted for the fabrication of scaffolds, including solvent casting, particle leaching, the creation of polymeric sponges, the use of spacer materials, freeze-drying and the electrospinning technique [7]. However, the use of these techniques makes scaffold construction complex, especially when dealing with intricate geometry and uniform porosity distribution.

- Rapid prototyping: the conventional fabrication techniques are gradually giving way to 3D printing as it allows computer-aided design (CAD) [20]. The scaffolds produced through this technique can be controlled in terms of shape, morphology and porosity, ensuring the transport of nutrients, oxygen and metabolic wastes. In addition, they have adequate mechanical properties due to their layered structure and specific design. This technique is fully compatible with various materials, including polymers, metals, ceramics and composites. Within 3D printing technology, there are different methodologies such as selective laser sintering, stereolithography, bioprinting and fused deposition modelling. The basis of 3D printing is the use of computer data such as CAD, computed tomography, and magnetic resonance imaging [7]. These scaffolds are particularly used in bone TE. The main advantage of 3D printing techniques is the ability to build patient-tailored scaffolds, high speed, ability to produce complex structures, low error rate, and high accuracy [21].

Biodegradation rate: except for some permanent applications, scaffolds are designed to gradually degrade, with a dissolution rate specific to the type of tissue and application. Indeed,

once implanted, cells release enzymes capable of digesting the scaffold. The gradual degradation of the biomaterial must occur at a rate that the body can replace it with new tissue. In addition, the degradation process mustn't lead to the formation or release of toxic fractions [7]. From this point of view, synthetic polymers offer numerous advantages, since by modulating the components and monomers, it is possible to create a material with the desired degradation rate.

Mechanical properties: each organ and tissue in the human body possesses its own mechanical stability. This characteristic must be carefully considered during the scaffold design phase in order to ensure the desired performance and prevent failure or fracture during the implantation period [22]. Although the use of metallic or ceramic materials can greatly improve the mechanical properties of the scaffold, it is important to remember that cell adhesion, proliferation and vascularization are also critical to the success of the process.

Architecture: it represents a crucial parameter in the tissue's regeneration ability. Especially for long-term implants, the porosity of the scaffold creates an environment in which cells can penetrate, and the interconnection of pores ensures nutrient flow and waste removal. In particular, pore architecture directly influences the adhesion of cells to the support structure and their penetration throughout the matrix. The four main factors to consider in scaffold design are pore size, total porosity, interconnection, and surface topography [7].
Currently, an interconnected porosity greater than 70 % is recommended, which promotes cell infiltration, migration, and proliferation [23] without unduly compromising mechanical properties. Pore size becomes particularly significant in the context of connective tissue regeneration, especially bone regeneration. Cellular interactions, such as adhesion, migration, and orientation, can be optimized when structure pores are in the range of 1-20 µm. In addition, pores in the range of 100-1000 µm are essential to ensure the flow of nutrients and the removal of wastes needed to feed cells and promote their proliferation [7]. Further research has shown the benefit of mesoporous structures (2-50 nm) in significantly increasing bioactivity.

Today, the greatest challenge for CTE and ATE is the development of advanced technologies capable of creating scaffolds that precisely replicate the structure of natural tissues. Depending on the application, the ideal scaffold must possess all the properties mentioned above. Over the years, various manufacturing techniques have been developed, with particular emphasis on 3D printers.
However, despite the progress made in the development of new materials, the use of natural solutions such as ECM, collagen, chitosan and alginate still remains an excellent solution. These materials are perfectly biocompatible and suitable for their respective applications.

BIOMOLECULES AND INDUCTIVE SIGNAL

The third key component of CTE consists of all those molecules that regulate the growth and development of cells, promoting their proliferation and differentiation. These substances may be already incorporated into the starting scaffold or added during tissue formation.
They mainly include morphogens and regulatory biomolecules.

1. **Morphogens:** are substances that influence cell proliferation and differentiation. These molecules, secreted and involved in pattern formation in developing tissues, are crucial for both embryonic development and adult tissue repair. Morphogens act across concentration gradients, inducing different cellular responses depending on their local concentration [24].

2. **Regulatory biomolecules:** this group includes differentiation, growth and cytokine factors, which are released by various cell types in different ways, such as endocrine, autocrine, paracrine, juxtacrine or intracrine. These molecules act on one or more target cells to trigger a specific response. When a growth factor binds to a receptor on target cells, an intracellular signal transduction system is activated that ultimately produces a biological response. These ligand-receptor interactions are highly specific and can range from simple cellular ligand-receptor binding to more complex interactions involving one or more ligands binding to one or more receptors.

Nowadays, the most widely used biomolecules in CTE are as follows:

- Epidermal growth factors (EGFs): promote the proliferation and differentiation of epidermal and epithelial cells.
- Fibroblast growth factors (FGFs): stimulate the proliferation of mesenchymal cells, chondrocytes and osteoblasts.
- Platelet-derived growth factors (PDGFs): promote the proliferation of mesenchymal cells, osteoblasts and fibroblasts.
- Insulin-like growth factors (IGFs): promote the proliferation and differentiation of osteoprogenitor cells.
- Transforming growth factor beta (TGF-ß): stimulate the proliferation of undifferentiated mesenchymal cells.
- Bone morphogenetic proteins (BMPs): promote the differentiation of mesenchymal cells into chondrocytes and osteoblasts osteoprogenitor cells into osteoblasts influences embryonic development.

1.1.2 Cell and Gene Teraphy

Cell Therapy (CT) and Gene Therapy (GT) both constitute advanced branches of RM that exploit distinct approaches to treating disease and improving human health.

These methodologies represent significant innovations in the field of RM, especially as they enable the development of therapies tailored to specific patient needs.
The next section will examine the main differences between the two techniques, as both involve cells, but at different levels.

Cell Therapy

CT involves the direct use of cells as a therapeutic modality, thus it is distinguished from CTE by its typical scaffold-free nature (or structural support). Among the most frequently used cells are stem, immune, and hematopoietic cells, as well as other specialized cells. Commonly, these cells are introduced by direct injection into the human body. Examples of CT applications include the use of stem cells to treat bone marrow lesions, the use of hematopoietic cells to address leukaemias and lymphomas, and the use of immune cells to treat certain forms of neoplasms.
Nevertheless, it should be emphasized that this disciplinary field is in its infancy thus it's constantly evolving. Currently, research is directed toward a deeper understanding of the action mechanisms of the used cells, as well as the evaluation of their long-term safety and efficacy.
Among the main treatment methodologies, the following stand out:

1. **Injection of the cell suspension:** direct injection into the venous system or tissues is the most commonly used method, due to its ease of management, speed of execution and minimal invasiveness, as well as reduced surgical complexity. However, it has demonstrated limited cell retention at the target site [12].

2. **The dense cell sheet construct** represents an innovative technique that promotes self-assembly of microtissues due to efficient interactions between individual cells and between cell and matrix. This approach is based on thermoresponsive cell culture technology, which allows the manipulation and harvesting of cell sheets without the need for enzymatic digestion. By preserving the crucial connections between cells and the ECM, the cell sheet offers a promising strategy for creating functional and structured tissues for CT and RM applications [25].

3. **Cellular spheroids:** represent another approach of the scaffold-free cell-based strategy, which allows for a three-dimensional construction of cells in contact with each other and communicating with the surrounding matrix [25].

4. **Bioprinting:** is an innovative technology widely used in CT. This method involves placing different types of cells in a pattern similar to native tissue, producing a matrix suitable for tissue construction. However, the main drawback lies in the need for a considerable amount of cells to create a three-dimensional, extracellular matrix-rich tissue equivalent within a commercially viable time frame. This limitation is a significant obstacle to the clinical translation and commercialization of this type of strategy.

Despite the different above-mentioned applications, all CT techniques include a succession of five main steps, which are shown in Fig. 1.3.

Figure 1.3: Fundamental steps in a CT-based technique [F3]

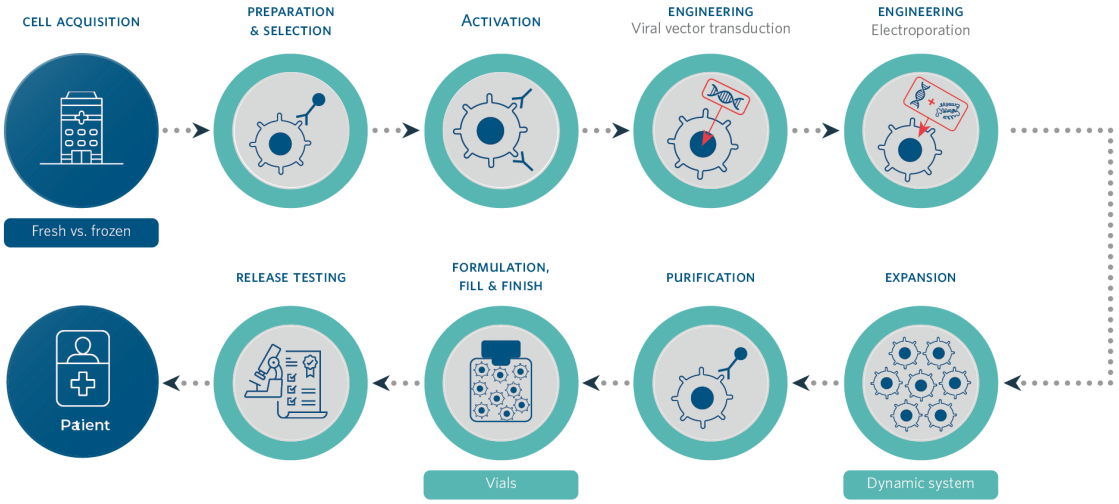
Resuming, CT represents a new branch of RM, with the potential to provide solutions for a wide range of pathologies but also for tissue regeneration. However, many challenges and issues remain, particularly concerning limited cell retention at target sites and the need to develop large-scale manufacturing processes.

Gene Therapy

GT focuses on manipulating the genetic material contained within cells in order to treat or prevent disease. In this case, therapy involves the introduction, modification or correction of genes within the body to repair or replace gene defects [26].
In Fig. 1.4 are reported the main steps to be performed in a GT-based technique.

GT is divided into somatic and germline. In the former case the genetic changes will not be passed on to the offspring instead in the latter case they could be transmitted and thus inherited by the offspring.
The most commonly used vectors able to transport genetic material within cells are the modified viruses and the nonviral vectors.
Modified viruses are rendered harmless by removing their viral genes and replacing them with therapeutic genes. The process is known as gene addition. Although viral vectors are designed to be nonreplicative, they can still provoke immune responses and adverse reactions. Among the most widely used are:

Figure 1.4: Fundamental steps in a GT-based technique [F4]



- **Retroviruses:** are RNA viruses that use an enzyme called reverse transcriptase to convert their RNA into DNA, which can then be integrated into the genome of the host cell. This integration process can make GT permanent, as the inserted genetic material becomes part of the host cell's DNA and is inherited by its daughter cells. They are widely used, especially for inherited diseases such as severe combined immunodeficiency and thalassemia.
- **Adenoviruses:** are DNA viruses that can infect a wide range of human cells, but do not integrate their genetic material into the host genome. This means that GT with adenoviruses is often short-lived and requires repeated dosing. They are mainly used in GTs for cancer and genetic diseases.
- **Adeno-Associated Viruses (AAV):** are DNA viruses that can integrate their genetic material into the genome of the host cell in a relatively stable manner without causing significant disease. However, their ability to carry DNA is limited. They are widely used to treat inherited diseases such as Duchenne muscular dystrophy and Parkinson's disease.
- **Herpes simplex viruses (HSV):** are DNA viruses able to infect nerve cells and establish themselves latently in their genome. This characteristic has been exploited to develop GT vectors that can carry therapeutic genes into nerve cells. HSVs are being studied primarily for GT of cancer and neurological diseases.

In contrast, the second macro category of vectors consists in non-viral carriers. They offer several advantages such as increased safety and decreased immune response, although they may have

complications related to delivery efficiency and gene expression [26].
The most studied and used are:

- **Plasmids:** they are circular DNA molecules that are introduced into cells to express specific therapeutic genes. They can be designed to include promoters and other regulatory sequences to control the expression of the therapeutic gene. After being introduced into cells, plasmids can persist or integrate into the genome of the host cell. Once inside the cell, the therapeutic gene can be transcribed and translated to produce the desired protein. They are widely used in GT for correction of genetic defects, production of therapeutic proteins, and stimulation of immune response against cancer.
- **Lipid nanoparticles:** consist of lipids that self-assemble into spherical nanoparticles. They may also include other components such as polymers or peptides to enhance their stability and transport capacity. Lipid nanoparticles are loaded with genetic material such as DNA or RNA and used to transport these genes into target cells. Once inside the cell, the genetic material can be released and used for therapeutic gene expression. They are mainly used for therapeutic drug and gene delivery, including RNA-based vaccines and GTs for cancer and genetic diseases.
- **Polymeric nanoparticles:** consist of synthetic or natural polymers that can be designed to condense and protect genetic material. They work in the same way as lipid nanoparticle carriers.

GT is an extremely young and therefore evolving branch of RM, but it promises to treat a wide range of genetic and oncological diseases such as immunodeficiency, haemophilia, muscular dystrophy, and some cancers [27]. The ultimate frontier is controlled gene expression, potentially allowing specific genes to be turned on or off at appropriate times and places.

1.2 CELLULAR PROGRAMMING

Cellular Programming (CP) constitutes an interdisciplinary field resulting from the fusion of biology and engineering. It is fundamental in TE and RM applications. It consists in influencing cell behaviour through the use of external stimuli such as biochemical, mechanical, electrical or light signals. These inputs are applied to modulate specific cellular functions or biological processes.
In the section below, the various types of biostimulation will be discussed in detail, followed by the main cellular modulations.

1.2.1 Biostimulation

The term biostimulation refers to all those methods used as inputs in CP, with the ultimate goal of influencing cell behaviour. The main stimuli nowadays used in CP are:

- **Chemical stimuli:** this category includes molecules and chemical substances such as growth factors, cytokines, signal molecules and pharmacological agents. They can regulate cell growth, differentiation, immune responses, cell signal transmission, and modify cellular metabolism. These substances can be added during the tissue formation or may be incorporated into the initial scaffold. Hydrogels are a particular application, in fact due to their structure, they can slowly release substances and growth factors capable of influencing cells' behaviour.

- **Mechanical stimuli:** the use of specialized devices and platforms capable of generating mechanical stimuli is crucial for proper cell behaviour, especially for some tissues, such as muscle or generally connective tissue. Cells may be subjected to stretching, compression, fluid flow, or tensile forces. Such stimuli influence cell morphology, orientation, arrangement and migration. Also in the use of mechanical stimuli, the employment of appropriate scaffolds is crucial to provide an environment that influences cell arrangement and interaction, aiming for a structure and mechanical properties similar to those of native tissue.

- **Electrical stimuli:** this is primarily referred to as electrostimulation, which involves the application of electric fields or electric current to modulate cell functions. Through specific devices equipped with electrodes, constant or variable electric fields but also short or pulsatile electric pulses can be applied. They can influence cell polarization, migration, communication or uptake of external molecules such as drugs or genes in the GT. Some cell types also are excitable, thus these stimuli can also be used to induce contractions or modulate the contractile activity of muscle or nerve tissue.

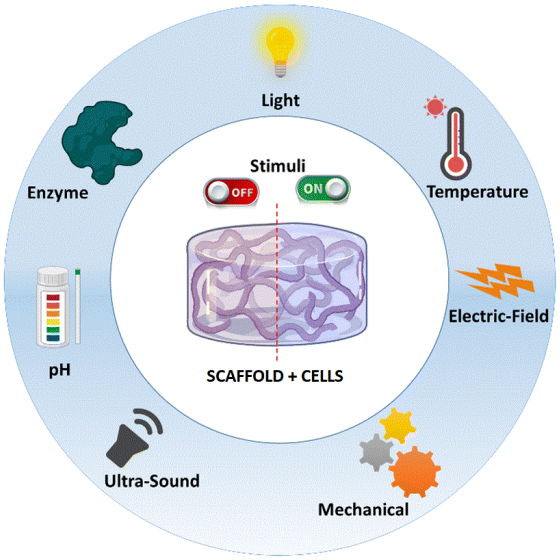
- **Light stimuli:** the term photobiomodulation refers to any approach that uses light at specific wavelengths that can penetrate tissues and interact with cells to change their behaviour. Lights with different wavelengths can be used, such as infrared, ultraviolet but also visible. In general, light interacts with cells by stimulating ATP and energy production (essential for tissue healing and regeneration) but also by modifying gene expression. Light therapy can have anti-inflammatory effects, reducing inflammation in tissues and promoting healing.

In Fig. 1.5 are summarized the most widely used biostimulation techniques in CP.

1.2.2 Cellular Modulations

Cellular modulations include all possible consequences and cellular responses induced by the stimuli mentioned above. In the context of RM and TE, a combination of stimuli is usually used so that each benefit is positively summed and the final desired effect is achieved.
The main types of cellular modulation are reported below.

Figure 1.5: Types of biostimulation used in CP [F5]



Cell differentiation

The cell differentiation process indicates the maturation of a cell from a primitive or undifferentiated state to a mature and differentiated form. This process involves changing the contents of the cell nucleus, in fact cell differentiation is obtained by the activation and deactivation of specific genes. The cell acquires different characters and specialized functions in order to achieve a division of tasks and functions typical of a multicellular organism. However, even today, most of the stimulus-related differentiation processes are not completely understood.
Differentiation occurs numerous times during the development of a multicellular organism, especially at the embryonic stage when it goes from a simple zygote to a complex system of tissues and cells of different types. However, differentiation also continues during adulthood as the body contains adult stem cells that can divide and create fully differentiated daughter cells. This process happens continuously during tissue repair and normal cell turnover.
As previously mentioned, in TE and RM the most commonly used cells are stem cells, a particular type whose function within the body is not yet well defined. They remain immature until a stimulus intervenes which causes them to differentiate into specialized cells. This normally occurs within our bodies, but if extracted, those cells can be cultured and then differentiated in vitro. In this case, using appropriate external stimuli of various kinds, they can be converted into different types of tissues, such as nerve, muscle, or bone, and then implanted.

Cell adhesion

Cell adhesion is the process by which cells bind to other cells, the ECM or any other type of artificial or natural substrate. This is essential since it influences tissue structure, cell communi-

cation, morphology and cellular functions [28].
Cell adhesion is provided by Cell Adhesion Molecules (CAMs), which are a category of cell surface proteins that allow cells to bind to each other and with the environment. CAMs allow forces and movement to be generated, forming the "molecular glue". CAMs are generally transmembrane receptors and are composed of three domains: an intracellular domain that communicates with the cytoskeleton, a transmembrane domain, and an extracellular domain. CAMs are divided into 4 main families:

1. **Immunoglobulin (IG):** IGs are a macro category of CAMs involved in homophilic and heterophilic binding and can bind integrins or different types of IGs. Molecules in this family play crucial roles in cell adhesion, migration, and neuronal synapse formation, such as neural cell adhesion molecules.

2. **Integrins:** are one of the major classes of receptors for ECM, ensuring the interaction between cell and ECM in collagen, fibrinogen, fibronectin, and vitronectin. They allow essential links between the extracellular environment and the intracellular signalling pathways. Integrins act as bridges between the cell cytoskeleton and the ECM, facilitating processes such as cell migration, adhesion, and cell-matrix communication [29].

3. **Cadherins:** are homophilic glycoproteins with a specific extracellular domain called extracellular cadherin domains (ECDs). They are gifts essential for cellular adhesion. Cadherins are calcium-dependent adhesion molecules involved in cell adhesion between cells of the same type. They play a key role in tissue formation during the development and maintenance of tissue structure.

4. **Selectins:** are a family of heterophilic CAMs. They have several functions, but are of extreme importance in the immune system, as they help white blood cell homing and trafficking. Selectins are involved in the adhesion of immune system cells (such as leukocytes) to blood vessel endothelial cells. This interaction is crucial for leukocyte recruitment during inflammation and immune response.

In TERM, cell adhesion is a crucial process because the main goal is to create functional tissues to replace or repair diseased or damaged ones. Cells must adhere not only to the scaffold but also to each other, forming a cohesive structure with desired properties. Proper adhesion also ensures cell survival and viability. In addition, once implanted in vivo, the tissue must integrate seamlessly with the surrounding tissue. This integration ensures that new cells can adhere to and communicate with host cells.
To improve cell adhesion on a scaffold, it is therefore necessary to adopt several strategies such as the use of surface modifications (like the addition of proteins or adhesive peptides), proper scaffold topography, and also the introduction of specific ligands. This is referred to as functionalization or decoration of the scaffold with specific ligands that bind to receptors on the cell surface. The optimal approach varies depending on the type of tissue, the specific needs and the purpose of the scaffold.

Cell migration

Cell migration is a central process in the maintenance of homeostasis and the development of multicellular organisms. Numerous actions such as tissue formation, wound healing, and immune reactions require the movement of cells in appropriate directions. Cells migrate in response to specific chemical or mechanical signals, which may be naturally produced by our body or sent from outside.
In general, body tissues are characterized by high viscosity and low Reynolds number. Cells must therefore produce forces to enable their movement. The latter is made possible by several mechanisms. The main ones are:

1. **Flagella or cilia:** many prokaryotes, as well as spermatozoa, use flagella or cilia for their propulsive movement. Flagella are long, thin structures protruding from the surface, mainly consisting of a flagellin protein. These flagella rotate like helices or propellants, allowing the cell to move through the surrounding fluid. The rotation of the flagella is powered by a molecular motor at the level of the cell membrane, which uses chemical energy to produce the rotary motion. This rotation creates a drive that allows the cells to move in the direction of the flagellum or change direction.

2. **Morphological alteration of the cytoskeleton:** eukaryotic cells move through dynamic extension and shrinkage of the cytoskeleton, particularly actin filaments. This process involves the extension of protrusions, such as pseudopodia or filopodia, from the front of the cell and the retraction of the back. Cytoskeleton proteins, such as actin and myosin, play a key role in generating pulling forces and moving the cell. Nowadays, scientists also talk about modification of the fluid mosaic pattern of the cell membrane. This suggests that the extension of the leading edge occurs primarily by the addition of a membrane at the front of the cell.

In TERM, cell migration is a fundamental process, as it allows cells to distribute throughout the scaffold, resulting in a compact final tissue with desired structural properties.
Promoting cell migration requires acting on the same parameters as cell adhesion. In fact, these two phenomena are complementary in that good adhesion provides the initial stability and connections necessary for cells to move and migrate on a substrate. On the other hand, cells to migrate, must overcome the adhesion forces. This trade-off in TERM is achieved through the precise regulation of adhesion molecules and their interactions with the substrate or scaffold.

Programmed cell death

Programmed cell death is a natural or induced mechanism of the cell that leads to self-destruction resulting in the death of the cell itself. It allows the preservation of the functionality of the entire organism at the expense of the individual cell (so-called altruistic suicide). It is a fundamental process by which the organism is shaped during embryogenesis and persists in the adult as

necessary to destroy cells that pose a danger to the integrity of the organism. Indeed, it occurs naturally in cases such as cells with damaged DNA, cancer cells, and virus-infected cells.
There are two types of apoptosis: that by an intrinsic pathway, initiated by problems within the cell (such as DNA damage, oxidative stress, mitochondrial damage, and lack of growth factors) and by an extrinsic pathway, initiated by signals coming from the external environment (biostimulation).
Thus, in TERM applications, it is possible to induce cell death by applying signals and molecules that induce apoptosis directly into the scaffold or during tissue generation. Examples are the use of pro-apoptotic proteins or more commonly the use of radiation (chemotherapeutics) to destroy cancer cells [30].

1.3 COLLAGEN

The term collagen is derived from the Greek $\kappa o\lambda\lambda\alpha$, meaning glue and the suffix $\gamma\epsilon\nu$, producer. In fact, its main function is to act as a glue for various connective tissues, giving them structure and support.
Collagen constitutes the main structural protein of connective tissue in animals, accounting for about 25 to 35% of the total protein content in mammals. In humans, it makes up about 6% of body weight, one-third of the total amount of protein, and three-quarters of the dry weight of skin. It is also the main component of the ECM [31].
Present mainly in connective tissues such as bone, ligaments, tendons, cartilage, and skin, collagen is also found in the cornea, digestive system, blood vessels, and teeth [32].
Collagen plays a key role in tissue architecture and integrity, mediates a wide range of cell-to-cell and cell-to-ECM interactions, and participates in several biological processes. Its presence can modulate cell adhesion and migration, cell growth and differentiation, morphogenesis, and tissue remodelling. It is also critical in wound repair, blood coagulation, and platelet adhesion.
Collagen can take on different mineralisation levels, thus determining its stiffness or flexibility. Based on this, collagen tissue can be rigid, as in bones, or flexible, as in tendons, but it can also occur in an intermediate state as in the case of cartilage.
Collagen is an extensively used material in TERM because being an essential and natural component of the ECM it can degrade easily, has strong plasticity, great tensile strength and a low immunogeneticity. However, pure collagen alone does not yield satisfactory experimental results [33]. Therefore, it is often admixed or decorated with other substances or scaffolds in order to give it mechanical and physical properties suitable for the specific use.
The part below will explore the many molecular structures of collagen, the different types and their functions, and the main applications in biomedical fields.

1.3.1 Molecular Structure

As previously explained, collagen is a structural protein, thus as all the other proteins it has a specific primary, secondary, tertiary and quaternary structure.

Primary structure

It represents the specific sequence of amino acids that make up each polypeptide chain. The distinguishing feature of the primary structure of collagen is the common presence of a repetitive sequence of three amino acids described as "Gly-X-Y."

- **Gly:** glycine is the most common amino acid in the primary structure of collagen and accounts for nearly one-third of all amino acids present. Its small size allows it to fit in the space between polypeptide chains, providing the typical tertiary triple-helix structure.
- **X and Y:** they represent any amino acid, but are often referred to proline and hydroxyproline. They are key amino acids that confer flexibility and stability to the triple helix structure of collagen and are important for the formation of intra-chain covalent bonds that stabilize the structure.

The Tab. 1.1 shows all the amino acids with their relative percentages found in the primary structure of type I collagen (the most abundant). It is worth noting that the amino acid hydroxyproline is uniquely present in collagen.

Secondary structure

Collagen is formed by joining multiple polypeptide chains together. Each chain forms a helix with a left-handed trend, similar to the classical alpha-helix. This is made possible by the recurrent presence of glycine and intramolecular hydrogen bonds.
In collagen, we can find two main types of chains, $\alpha 1$ and $\alpha 2$. They are very similar to each other in structural terms but differ slightly in their amino acid composition and gene expression. Each type of collagen is formed by a different pairing of alpha chains.

Tertiary structure

The typical tertiary structure of collagen, as well as the most stable, is the triple-stranded helix. In this arrangement, the structural unit is called tropocollagen and has a molecular weight of about 285-300 kDa. It consists of three polypeptide chains with a left-handed trend that aggregate to form a very narrow right-handed triple helix, measuring about 300nm in length with a diameter of 1.5nm. This triple-stranded arrangement gives collagen remarkable strength and structural stability.
This arrangement is mainly dictated by hydrogen bonds between the amino acids of the polypeptide chains. These bonds contribute to the stability of the triple-stranded structure.

Amino acid	3 letter code	Abundancy (%)
Glycine	Gly	26.7
Proline-Hydroxyproline	Pro-Hpro	19
Alanine	Ala	9.5
Glutamic Acid	Glu	5.1
Arginine	Arg	4.8
Aspartic Acid	Asp	4.5
Serine	Ser	4.1
Lysine	Lys	3.9
Glutamine	Gln	3.3
Leucine	Leu	3.3
Valine	Val	3.2
Threonine	Thr	3.1
Phenylalanine	Phe	1.8
Isoleucine	Ile	1.6
Asparagine	Asn	1.2
Cysteine	Cys	1.2
Methionine	Met	0.9
Tyrosine	Tyr	0.9
Histidine	His	0.6
Tryptophan	Trp	0.4

Table 1.1: Type I collagen amino acids and their percentage

In addition, the various chains are also linked by cross-links, special covalent bonds that are established between two lysines or allysines. Cross-links give collagen great mechanical and tensile strength as well as high chemical stability. The amount and type of cross-links vary with tissue type and age.

Quaternary structure

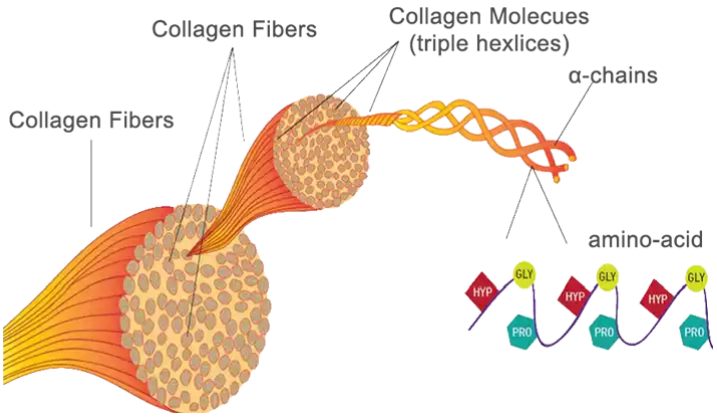
Tropocollagen, the structural unit, tends to self-assemble into fibrils according to extremely precise structures. Fibrils usually appear as long and thin and aggregate together to form larger structures, imparting strength and structural support to tissues. In some cases, collagen fibrils aggregate into larger bundles, forming collagen fibres, but they can also interact with other proteins or molecules in the ECM to form networks.

Resuming, in Fig. 1.6 is possible to distinguish the four different structures of the collagen.

1.3.2 Collagen Types

28 distinct types of collagen have currently been identified, all characterized by the presence of at least one triple helical domain. This structural feature can vary significantly within the

Figure 1.6: Collagen structures [F6]



molecule, accounting for 96% of the structure in the case of type I collagen, down to a minimum of 10% in type XII collagen.
The diversity among the various families is dictated not only by the existence of numerous alpha chains (the most important $\alpha 1$ and $\alpha 2$) but also by several molecular isoforms, supramolecular structures and the use of alternative promoters and splicing. The main and most abundant types of collagen are the following:

- **Type I collagen:** is the most common form of collagen in the human body, accounting for about 90% of the total amount. It consists of three polypeptide chains $[\alpha 1_2, \alpha 2]$ arranged in a triple-helix structure. It is part of major connective tissues, such as skin, tendons, bone, cartilage, adipose, and cornea. It provides structural strength and support, contributes to organ biomechanics, and participates in the wound-healing process by providing a structural matrix for cell migration and new tissue deposition.

- **Type II collagen:** forms cartilage, intervertebral discs, and the vitreous body. It consists of three polypeptide chains $[\alpha 1_3]$ arranged in a triple-helix structure and is mainly present in articular cartilage, increasing its compressive strength and shock absorption capacity. It provides structural support and stability to joints, enabling them to withstand the forces generated by movement and physical activity. Compared with type I, type II collagen is less prevalent in the human body.

- **Type III collagen:** contributes to the structure of blood vessels and capillaries, providing them with elasticity and strength. It is also the collagen found in granulation tissue. It consists of three polypeptide chains $[\alpha 1_3]$, which have a greater distance between its molecules than type I collagen. This gives type III collagen greater flexibility. It is found primarily in tissues that require elasticity and flexibility, such as skin, blood vessels, and smooth muscle. It also plays a role in wound healing as it is produced quickly and in large quantities before type I collagen, from which it is later replaced due to its higher resistance.

In general, each of the 28 types of collagen has its specific structure and function, so it can be found in certain types of tissues. The 28 types can be easily condensed into 3 macro categories:

1. **Fibril-forming collagens:** this category includes all types of collagen-forming fibrils, beaded filaments and anchoring fibrils. Fibrils are generally formed by different types of collagen and are therefore called heterotypic. Collagen fibrils range in diameter from approximately 15nm up to 500nm [34] and show a banding pattern with a periodicity of 64-67nm. Fibrils confer strength and support to connective tissues, such as skin, bone, tendons, and ligaments.

2. **Fibril-associated collagens:** they do not properly form fibrils, but are covalently associated with the surface of the former, forming a large bridge linking structure. They mediate the organization of fibrils by helping them to assume an ordered structure.

3. **Network-forming collagens:** this category includes the various types of collagens that form three-dimensional networks within tissues. Type IV collagen is the main representative of this category and is found in the basement membrane, where it plays an important role in filtration and structural support of organs. It is essential, in fact it acts as a barrier and support for epithelial tissues.

1.3.3 Extraction and Applications in Biomedical Field

The main source of collagen used in the biomedical field comes from various tissues of animal origin, particularly bone, tendons, and lungs from swine, equines, or cattle [35].
Collagen is inherently characterized by low immunogenicity, which can be further reduced by the use of specific proteases or through the process of crosslinking, to screen antigenic sites.
Collagen molecules can be extracted and purified using different techniques such as acid treatments (dilute acetic acid), alkaline treatments (sodium hydroxide solutions) or protein hydrolysis followed by neutral salt treatment, dialysis, precipitation, and centrifugation.
To be then usable in the biomedical field, collagen must be processed with appropriate physicochemical treatments such as decellularization, demineralization, cross-linking, dehydration, and lyophilization.
Collagen is an extensively used biomaterial in TERM because it is highly biocompatible, biologically active, easily degradable, promotes osteogenesis and adhesion, and has low immunogenicity. The main disadvantages are related to its poor mechanical properties (such as mechanical strength), which limits its use in some cases. To overcome this, it is often necessary to supplement or combine collagen with additional structural support (such as a titanium scaffold) to improve its performance. In this case, it is referred to as "improved collagen".
All these properties, combined with versatility, ease of use and availability, make collagen one of the most widely used materials in TE of connective tissues, especially in bone TE. In fact, some research shows that collagen can promote the differentiation and proliferation of bone mesenchy-

mal stem cells (BMSCs) and osteoclasts, thereby promoting osteogenesis.
Currently, in TERM, collagen is used in the form of:

- **Collagen-based hydrogels:** hydrogels are crosslinked colloidal compounds with a highly hydrated three-dimensional structure. They enjoy high permeability to oxygen, metabolites and nutrients, and adhesive and sealing properties. They can be used as scaffolds in TE but also as drug delivery systems. The main disadvantages are related to their poor mechanical properties. In fact, hydrogels cannot withstand pressures such as bone pressure, so they are mainly used as wraps for cells or drugs.

- **Collagen-based scaffolds:** collagen scaffolds play a key role in TE (both CTE and ATE), especially bone TE. In fact, they perfectly mimic the native structure of bone tissue, having the right porosity, the correct pore size and some amino acid sequences with high adhesive properties. As with hydrogels, scaffolds composed only of natural collagen do not completely fulfil the requirements of mechanical strength in vivo so they sometimes need to be fortified with metals such as titanium. In general, they show good regeneration for bone tissues but also for cartilage defects.

1.4 BIOACTIVE PEPTIDES

Bioactive Peptides (BPs) are organic substances formed by a chain of amino acids covalently linked together by amide or peptide bonds. Unlike proteins, which are polypeptides with a high molecular weight, BPs generally possess a low molecular weight. They are protein fragments with a number of amino acid residues usually between 2 and 20, but still always lower than 50 [36]. This is precisely why their molecular mass is generally between 0.4-2 kDa. Only in rare cases, longer-chain BPs have been confirmed, as in the case of lunasin [36].
Although some of these peptides are found in a bare format, most of them are hidden in the inner chain of proteins [37]. An abundance of some amino acids, such as proline, arginine, lysine, and other hydrophobic residues can be found in the chains of BPs.

The activity and function of BPs are closely related to their structure, which means the amino acid composition, the type of N- and C-terminal amino acids, the length, the amino acid charge, and the hydrophobic/hydrophilic characteristics of the chain. BPs are capable of interacting with cell receptors, proteins or other biological molecules, inducing a biological or pharmacological response. They therefore have specific functions within a living organism or cell.

BPs play fundamental roles in the metabolic functions of living organisms and, consequently, in human health in general. They possess several significant functions, such as antioxidant, anti-inflammatory, anticancer, antimicrobial, immunomodulatory, and antihypertensive. They play a crucial role in human health by influencing the digestive, endocrine, cardiovascular, immune

and nervous systems. They can prevent microbial oxidation and degradation and act as mineral binders. They also act as potential modifiers, reducing the risk of many chronic diseases. This is precisely why they are considered the next generation of biologically active regulators [38].

From a structural point of view, there is no consensus on the architecture of BPs, in fact, each of them has a very specific sequence of amino acids, called motif. The various motifs can be divided according to their function into the following categories:

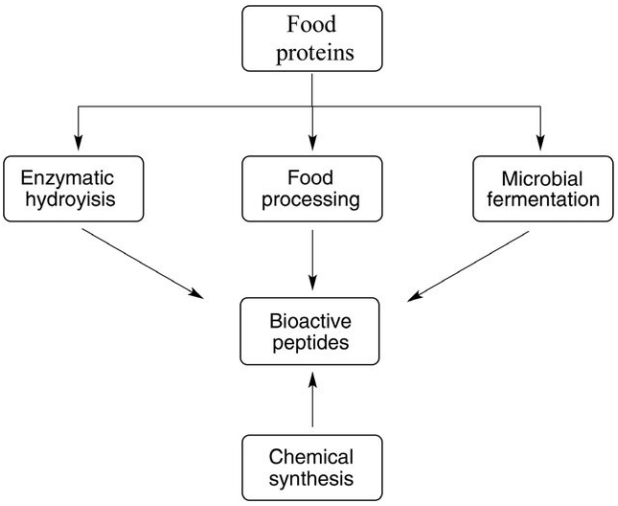
- **Recognition motifs:** specific sequences of amino acids that enable them to interact selectively and with high affinity with other biological molecules.
- **Binding or anchoring motifs:** allow peptides to bind selectively and specifically to target molecules, facilitating interactions and formation of functional complexes.
- **Localization motifs:** are crucial to ensure that molecules reach their target site and perform their biological functions effectively and specifically.
- **Motifs of biological activity:** determine the biological function of peptides, such as antimicrobial, anti-inflammatory or anticancer activity.
- **Signaling motifs:** may be recognized by proteins or receptors, which initiate a cascade of biochemical events culminating in a determined biological response.

BPs can be classified also on the basis of their related origin. There are endogenous peptides which are produced in different types of cells, such as neural, and immune cells or in various glands of the body, such as pancreas, liver, pituitary and adrenal glands. On the other hand there are the exogenous peptide, which enter the body from various sources, such as foods, dietary supplements and drugs.

From a therapeutic point of view, peptides offer numerous benefits that sometimes make them more useful than traditional drugs [39]. For example, BPs have more specialized activities on the target tissue, hence little or no toxic effects, and are effective even at low concentrations.
Nowadays, BPs are attracting continuous and growing interest in biomedical engineering, specifically in TERM. This is related to their beneficial and cyto-modulatory properties, as well as their easy availability. In fact, as reported in Fig. 1.7, they can be extracted directly from protein (usually derived from food) or in other cases chemically synthesised.

The main field of use of BPs in RM is the functionalization of biomaterials such as polymers, and hydrogels in order to impart specific bioactive properties to them, enhance interaction with cells, promote cell adhesion, and regulate immune response. It is thus possible to modulate the interaction between biomaterials, cells, and tissues.
Usually, in TERM, scaffolds are added in order to mimic molecular signals present in the natural

Figure 1.7: Main sources of BPs [F7]



cellular and tissue environment, thereby facilitating the formation and function of new tissues. Some of the main applications of BPs in TERM are described below:

- **Promote cell adhesion and proliferation:** BPs facilitate cell colonization of scaffolds. Examples include ECM mimetic peptides, such as RGD (arginine-glycine-aspartate). They are chemically bound to the surface of the scaffold and promote cell adhesion and spreading, promoting tissue regeneration and production.

- **Control cell differentiation:** some BPs can drive cell differentiation toward specific phenotypes, enabling the production of tissues with desired morphological and functional characteristics. For example, peptides such as BMP-2 (bone morphogenetic protein-2) mimic growth factor differentiation signals and promote differentiation of MSCs into bone cells, thereby facilitating bone regeneration [40].

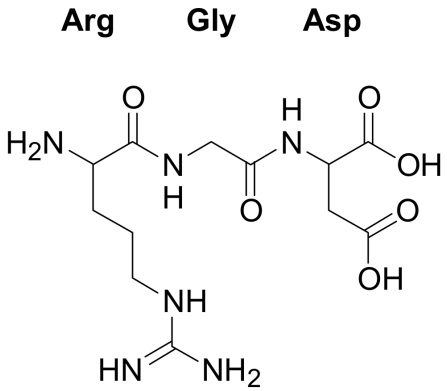
- **Promote blood vessel formation:** BPs can promote angiogenesis thereby improving perfusion and cell survival. For example, peptides such as VEGF (vascular endothelial growth factor) or FGF (fibroblast growth factor) mimic angiogenic signals therefore stimulating blood vessel growth and improving vascularization [41].

- **Modulation of the immune response:** BPs can modulate the local immune response, reducing inflammation and improving the biocompatibility of implantable biomaterials. For example, anti-inflammatory peptides, such as LL-37, can be incorporated into biomaterials to inhibit the production of inflammatory cytokines by reducing the adverse immune response [42].

1.4.1 RGD

RGD, from a chemical point of view, is a tripeptide, thus a molecule composed of three amino acids covalently linked together by peptide bond: arginine (R-Arg), glycine (G-Gly) and aspartic acid (D-Asp). The corresponding chemical structure is reported in Fig. 1.8.

Figure 1.8: RGD chemical structure [F8]



RGD is a naturally occurring peptide in several molecules such as fibronectin, laminin, fibrinogen, osteopontin, and vitronectin [43]. It functions as a signalling system in the cell surface, thus falling into the category of BP. In fact, numerous studies (discussed in detail later) have shown that RGD can enhance cell adhesion and spreading, particularly that of osteoblasts. At the same time, it promotes cell proliferation, osteoblast differentiation and mineralization.
RGD can be used in its primitive form (tripeptide) or can be found in a much longer amino acid sequence. It also exists in a cyclic form, which is currently considered more active than the linear form. In fact, it has a higher affinity for integrin receptors and is more resistant to proteolysis.

Nowadays RGD is one of the most effective and used BPs for stimulated cell adhesion, especially on synthetic surfaces [43]. The RGD peptide family is considered a specific recognition site for the integrin receptors [44]. The latter fall into the category of cell adhesion receptors and are key regulators of cell-cell and cell-extracellular microenvironment communication.
Integrins are located on the cell membrane and are composed of alpha and beta subunits. They perform the function of transmembrane receptors allowing the anchoring of cells to the ECM. They are also involved in other processes such as embryogenesis, cell differentiation, immune response, wound healing and hemostasis, proliferation, and cell apoptosis. Integrins also play a crucial role in bone and cartilage repair, contributing to the recruitment of MSCs to the site of defect for repair. They are essential for osteoblast survival and bone mineralization, helping to increase bone mineral density [43].
The binding process and interaction mechanism between RGDs and integrins is still poorly under-

stood at the molecular level, especially under natural conditions. Several simulations hypothesize that adhesion occurs in four main steps:

1. **Recognition of the RGD motif:** integrins recognize the RGD motif, which acts as a molecular label and signals for the cells, signalling the presence of binding sites to the ECM.

2. **Binding with integrins:** once the motif is recognized, interactions are established between the various binding sites of the two units. Some simulations have shown that the formation of a salt bridge between the RGD amino acid arginine and asparagine is the main mechanism of interaction. In addition, the various hydrogen bonds between the two units also play an important role in stabilization.

3. **Complex formation:** the interaction between the two units leads to the formation of a complex, which provides a solid basis for cell adhesion and stabilizes cell-substrate interactions.

4. **Activation of signalling pathways:** integrins activate intracellular signals that can influence various cellular processes.

Applications in biomedical field

Currently, RGD peptide plays an important role in the biomedical field, both as a coating material for implantable devices and as a biomolecule in TE.
In the former case, RGD is widely used to coat screws, nails, rods, and other implantable metal devices. These coatings have been shown to promote peri-implant bone formation, consequently leading to a significant increase in the bone-implant interface. RGD has been shown to further enhance cell density and migration in the surrounding microscopic environment. It not only increases tissue growth but also reduces the fibrous tissue formation at the interface [44].
In the second case, RGD is incorporated into scaffolds, especially synthetic or metallic ones, in order to enhance their biological properties such as cell adhesion and proliferation. It can also be bound to natural scaffolds, such as collagen or hyaluronic acid scaffolds, to further enhance their performance [45].

1.5 COLLAGEN FUNCTIONALIZATION

The term functionalization refers to all those processes aimed to modify the internal or surface properties of a material, giving it specific functionality or performance. In the biomedical field, functionalization of biomaterials is a very common practice, as it allows the design of advanced medical devices with desired properties.
In fact, through the functionalization, the following factors can be increased:

- **Biocompatibility:** by modifying the surface layers of a material with highly bioactive components, the risk of immune reactions and rejection can be reduced.
- **Cell adhesion and proliferation:** by introducing binding motifs and BPs, cells can easily adhere to the material and colonize it, promoting tissue regeneration and growth.
- **Mechanical properties:** by introducing certain compounds (usually metallic ones), the mechanical properties of the material can be improved, making it more or less elastic, strong, hard etc.
- **Controlled release of drugs or biomolecules:** in drug delivery systems, it's possible to modify the material surfaces by incorporating active biomolecules (such as drugs) and controlling their release.

There are several functionalization techniques. The main ones include chemical modification, thin layer deposition, use of biopolymers, conjugation of bioactive molecules, and addition of nanoparticles.

Within the macro category functionalization, we can distinguish two different techniques widely used in biomedical engineering and TERM. They are:

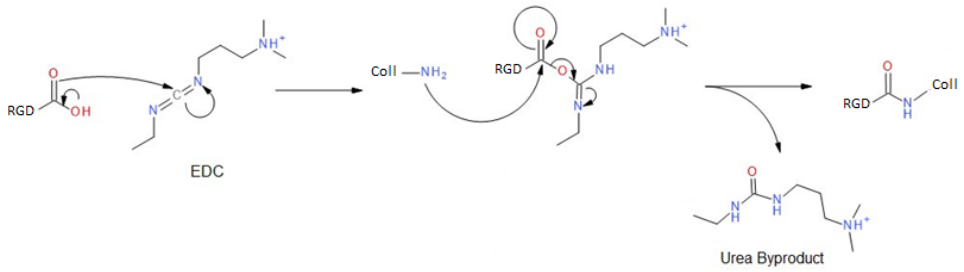
1. **Derivatization:** is the process by which a molecule is chemically altered by adding specific functional groups or modifying existing ones. It is widely used to modify the physicochemical and biological properties of a biomaterial.
2. **Decoration:** this technique usually involves the addition of components such as biomolecules, nanoparticles, patterns or structures in general, to improve performance and properties. They can be chemically bound or deposited on the surface of the material.

In this chapter, will be reported the most widely used techniques for collagen functionalization with BPs, in this specific case the RGD. It can improve the collagen standard properties such as biocompatibility, adhesion and cell proliferation [46].
Three are the methods for covalently binding RGD to collagen.

1.5.1 EDC

EDC, the acronym for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, is a hetero-bifunctional coupling agent widely used in cross-linking reactions between protein chains and therefore also in peptide bond formation.
EDC is able to activate the carboxyl groups of a peptide (such as RGD) both in an aqueous environment [47] and in certain buffers such as MES, 2-(N-morpholino) ethanesulfonic acid [48]. The EDC-RGD complex is then added to a collagen matrix or suspension. The activated peptide binds covalently via nucleophilic attachment to free amines in collagen residues (especially the

Figure 1.9: Collagen functionalization through EDC: reaction mechanism [F9]



highly reactive lysine).
The reaction occurs overnight, at low temperatures and possibly in an acid environment to avoid self-assembly of collagen fibres [48]. In fact, EDC is also used as a crosslinking agent for collagen. Urea, formed as a byproduct, must be removed in a subsequent step [47].

Studies show that the reaction yield can reach values up to 50-60% of the initial mass of added peptide [48]. In addition, in-vitro and in-vivo experiments show an increased cell adhesion and the maintenance (without particular alterations) of the chemical and physical native structure of collagen, preserving its mechanical properties [47,48]. The only disadvantage is the slight decrease in cell migration, mainly due to the high adhesion [48].

1.5.2 Sulfo-LC-SPDP

Sulfo-LC-SPDP, the acronym for sulfosuccinimidyl 6-[3-[3-(2-pyridyldithio)-propionamido] hexonate, is currently one of the most widely used compounds for the decoration of protein substrates, especially collagen [49-52].
It falls into the category of water-soluble and hetero bifunctional cross-linker (such as EDC) and succeeds in coupling collagen with various peptides (such as RGD) in a process involving three chain reactions (Fig. 1.10):

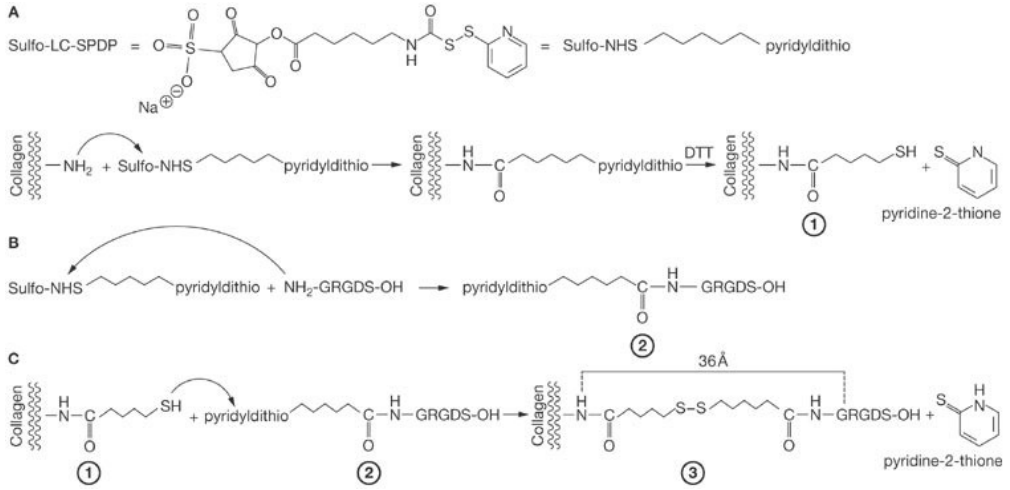
A Sulfo-LC-SPDP react separately with collagen-free amino groups at room temperature forming 1.

B Sulfo-LC-SPDP was separately coupled to the amino terminus of the RGD peptide producing 2.

C Compounds resulting from steps A and B react together to give the final product 3.

All the reactions are performed at neutral pH, and any excess of reagents or by-products must be removed. In addition, it has been shown that the use of a spacer arm sized between 30-40 Å(included in the backbone of the Sulfo-LC-SPDP cross-linker) optimises the RGD presentation and favourable for integrin-receptor clustering and activation [49,51,52]. High levels of peptide

Figure 1.10: Collagen functionalization through SULFO-LC-SPDP: reaction mechanism [F10]



functionalization can be achieved with this type of reaction, with a yield of about 70% [49].

Numerous studies show that the use of this particular cross-linker, combined with the introduction of a spacer arm between 30 and 40 Å, produces a decorated collagen with improved cell adhesion, survival, growth and differentiation [49,50,52]. In terms of Young modulus, physical and mechanical properties, RGD-decorated collagen does not differ from native or control collagen [51,52].
This technique is nowadays widely studied and used as it has achieved great results in myocardial TE, increasing the contractile level and performances of the collagen scaffolds seeded with MSCs.

1.5.3 Periodate Activation

Periodates are chemical compounds with brute formula IO_4- used to covalently bind peptides to matrices composed of hyaluronic acid, collagen and similar tissues [53-55].
Periodate, usually sodium periodate ($NaIO_4$), can create reactive aldehyde groups in collagen by mainly oxidizing the hydroxyproline residue [53-55]. The new aldehyde groups behave as an active site for peptide coupling. In this reaction, collagen, periodate, peptide and certain buffers (such as sodium acetate) react at room temperature for about 4 hours. Subsequently, the collagen sample is purified and sometimes lyophilized.
Several in vivo and in vitro studies show that collagen decorated with RGD by periodate oxidation has increased interaction with cells and improved cell adhesion if compared with pure collagen [53-55].
However, adhesion is not the only key factor for a successful engineered tissue. It has been shown how the use of periodate, which is an oxidizing compound, causes a decrease in the intensity of covalent bonds in the collagen matrix, destroying native structures [54,55]. In addition, inter-

connections between pores decrease, many pores disappear, and tension strength and rupture length also decrease significantly [54,55]. All these factors provoke a reduction in cell proliferation.

Thus combining the positive effect on cell adhesion and the negative effect on proliferation and mechanical properties, it's possible to affirm that the use of periodate does not increase overall collagen functionality. This is the reason why this method has been almost totally abandoned nowadays.

1.6 ANALYTICAL TECHNIQUES

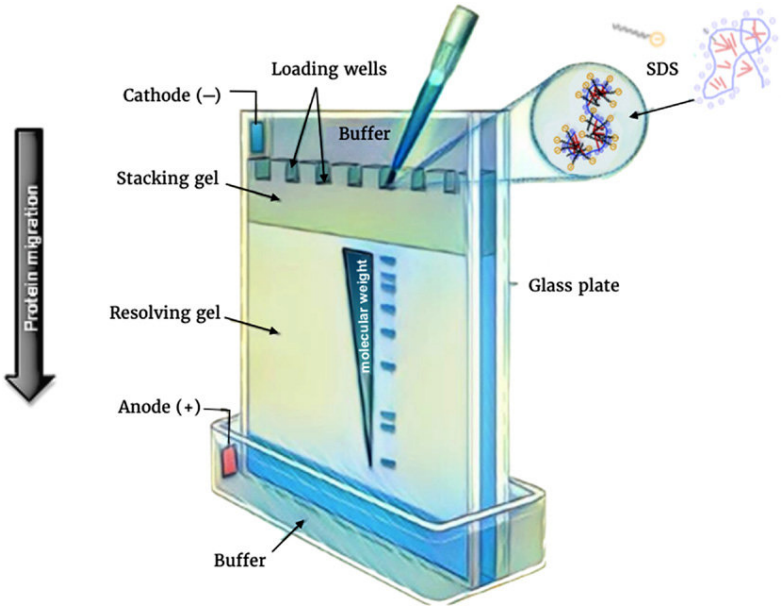
1.6.1 SDS PAGE

Electrophoresis is one of the most widely used laboratory techniques for separating molecules (especially proteic ones) according to their molecular weight. It exploits substances' natural or induced charge in conjunction with an electric field. Molecules that are naturally charged, such as nucleic acids that have phosphate groups, once immersed in an electric field will spontaneously tend to migrate to the opposite pole. In contrast, in the case of proteins, which do not have a uniform charge, it's necessary to bind ionic compounds to the protein chain, causing their subsequent migration.
SDS-PAGE, which stands for Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis, is a special type of electrophoresis performed on a polyacrylamide gel in the presence of sodium dodecyl sulfate. It is an analytical-qualitative technique that allows to determine with good accuracy the purity degree of the protein and its molecular weight. It is not a quantitative technique since the amount of protein that can be separated is about 5 to 25 µg per band. This does not allow its initial quantification.
As reported in Fig. 1.11 this technique is based on two main components: the SDS and the Polyacrylamide gel.

1. **SDS:** is a strongly anionic and denaturing detergent, able to react with protein compounds in a well-defined ratio. Thus it gives molecules uniform negative electrical charge, on average one negative charge for every two amino acid residues. Proteins will have a number of negative charges proportional to their molecular weight, more precisely about 45-50 charges for every 10 kDa. The proteins are also denatured by destroying almost all noncovalent interactions and therefore stretched into nearly linear chains. Subsequent separation occurs by the difference between molecular weights since the mass-to-charge ratio for each denatured protein remains constant.
   In addition, the use of reducing substances, such as dithiothreitol (DTT) causes the reduction of any disulfide bonds present between the cysteine residues.

2. **Polyacrylamide gel:** acts as a three-dimensional sieve allowing proteins to be separated based on their molecular weight. The gel consists of two essential parts:

Figure 1.11: SDS PAGE components and functioning [F11]



- Stacking gel: is the upper part of the gel and its function is to align inhomogeneous samples, allowing the starting of the protein migration from the same point.
- Running gel: is the lower part and its function is to separate proteins based on their molecular weight. It is mainly formed of an acrylamide polymer, whose concentration varies depending on the desired porosity: higher concentrations lead to smaller pore sizes, therefore capable of separating proteins with higher resolution. Polyacrylamide percentage values are normally between 3 and 15%.

The procedure is simple and fairly quick. The sample is prepared by adding SDS, any reducing agents (such as DTT) and dyes. Then it is loaded into the appropriate wells on the acrylamide gel. The gel is submerged in an appropriate running buffer, and supplying the device with direct current at constant power begins the protein migration toward the anode. The SDS-protein complexes are then separated according to their molecular weights by the molecular sieving effect of the polyacrylamide gel. This causes smaller proteins to migrate more rapidly through the gel, while larger proteins migrate more slowly.
At the end of the run, each protein will have travelled a distance in the gel inversely proportional to its molecular weight. In addition, the molecular weights of the various proteins can be determined by comparing them with some markers of known molecular weight previously loaded into another well.
Usually, protein compounds are not coloured, thus to observe the bands it's necessary to highlight them with dyes or fluorophores.

1.6.2 UV-VIS Spectrophotometry

UV-VIS spectrophotometry is an analytical technique commonly used in chemistry to study the interactions between the sample and radiation in the electromagnetic spectrum. When a photon contained in the UV-VIS spectrum interacts with a substance, it can cause absorption, reflection or refraction phenomena.
This technique uses wavelengths ranging from 10 nm and 760 nm. In this range, the valence electrons of compounds are used to be excited, causing the so-called atomic electron transition: the electron goes from a fundamental to an excited energy state. This phenomenon causes absorption of electromagnetic radiation, but it occurs only at certain wavelengths, which are characteristic of the bonds present in the molecule. It means that absorptions at certain wavelengths are characteristic of a particular chemical substance, and this is exploited to conduct qualitative and quantitative analyses.
A typical UV-VIS spectrum contains in the ordinates the absorbance or transmittance as a function of the various wavelengths, which are represented in the abscissae. The spectrum reports a series of peaks of different heights characteristic of the compound being analyzed.
The block diagram of a UV-VIS spectrophotometer (reported in Fig. 1.12) consists of the following components:

1. **Source:** produces the radiation in the UV-VIS spectrum. The most commonly used lamps are incandescent, deuterium, xenon and tungsten lamps.

2. **Monochromator:** is the device that separates the radiation into its different components. It allows the passage of wavelengths one at a time, isolating a narrow range of radiation. It consists of concave mirrors and reflection gratings.

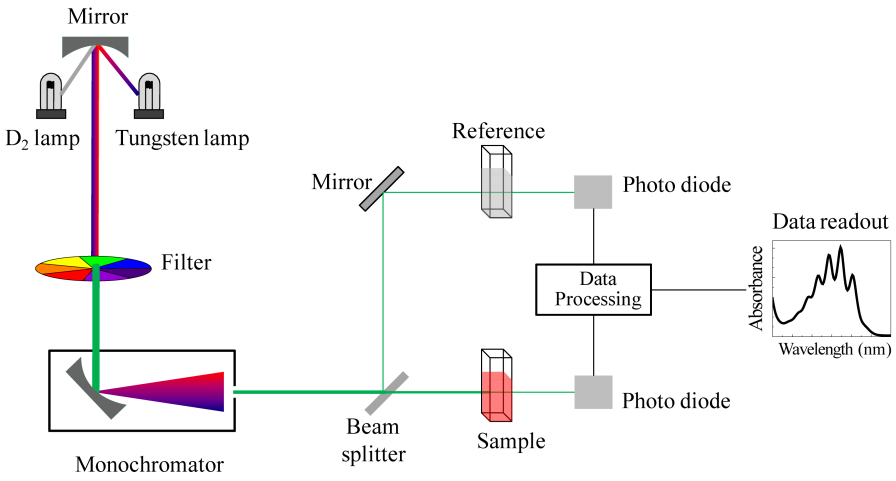
3. **Sample:** is usually contained in a parallelepiped-shaped container called cuvette. The cuvettes must be transparent to the working wavelength, thus they are composed of glass or plastic for the VIS and quartz for the UV. Part of the incident radiation will be absorbed by the sample, while the remainder will be transmitted arriving at the detector.

4. **Detector:** device that measures the radiation transmitted by the sample. It receives the signal and sends it to the data processing system (computer), which quantifies the absorbance or transmittance. The typically used detectors are vacuum and gas photocells, photomultipliers, photovoltaic cells, photoconductive cells, and photodiodes.

Nowadays, there are dual-beam UV-VIS spectrophotometers (Fig 1.12). In this case, once selected by the monochromator, the radiation is split by a mirror system and hits two cuvettes, one containing the sample and one the standard. The detector compares the two signals performing an analysis with less noise with respect single-beam devices.

These kinds of devices are mainly used for quantitative and qualitative analysis.

Figure 1.12: UV-VIS spectrophotometer block diagram [F12]



Quantitative analysis: these procedures can be performed only in certain concentration ranges in which the Lambert-Beer law (equation reported below) is valid and therefore there is linearity between concentration and absorbance.
The operator must know the substance under analysis and therefore also its absorption spectrum. Once the characteristic absorbance of the compound has been determined, the monochromator must be set to that wavelength. Then the calibration curve is constructed using the absorbances of samples of known concentration. Usually, 3 to 5 points are used to define the line. At this point, the sample of unknown concentration is analyzed, and once the absorbance is known, the concentration value can be determined using the Lambert-Beer equation.

$$A = \epsilon * l * C$$

where:

- A is the absorbance
- ϵ is the molar absorption coefficient
- l is the optical field length (nominal cuvette thickness is usually 1cm)
- C is the concentration

It's fundamental to consider that the reproducibility and accuracy of this technique depend on several factors such as operating conditions, sample processing, line construction etc.

Qualitative analysis: in this case, the unknown sample is subjected to a wide range of wavelengths and the absorbance is determined for each. An absorption spectrum characteristic of the sample is created. Then it is automatically compared with spectra of known substances contained in various databases to discover the unknown sample.
Qualitative analyses are not very common in professional UV-VIS spectrophotometry because the initial sample requires good purity and high concentrations.
For qualitative analysis, it is preferable to perform IR spectrophotometry, which is much more accurate and detailed.

1.6.3 Fluorimetry

Fluorimetry is a qualitative and quantitative chemical analysis based on the fluorescent properties of certain substances. Fluorescence is a property of some molecules to re-emit absorbed electromagnetic radiation at a longer wavelength, and therefore lower energy (in most cases).
As previously explained when radiation strikes a molecule it can excite it to a high-energy state. This phenomenon is followed by a decay process, which in most cases consists in damping, vibrational relaxation or internal conversion. In some cases, however, the excited electron returns to the fundamental level by passing through one or more intermediate-energy excited states. The last jump can emit light at a longer wavelength than the incident radiation, causing the phenomenon called fluorescence.
Fluorometers are devices that exploit precisely the fluorescent radiation emitted by the substance to determine its nature (and thus its chemical composition) or more simply its concentration.
The block diagram of a fluorometer (Fig. 1.13) consists of the following components:

1. **Light source:** emits the radiation necessary to excite the sample under examination. Usually consists of a xenon lamp with a continuous spectrum from 250 to 1000 nm.

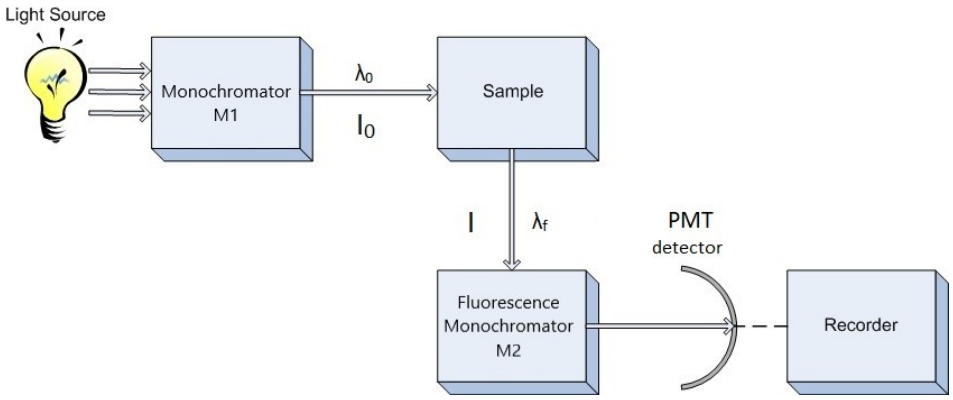
2. **Excitation monochromator (M1):** allows the light from the source to be selected at a specific wavelength.

3. **Sample holder:** the radiation strikes the sample usually contained in a test tube made of specific materials that do not interfere with the measurement.

4. **Emission monochromator (M2):** used to select the wavelength of radiation emitted by the sample to be sent to the detector. Both monochromators are formed by gratings, which allow to minimize the effects related to scattered light.

5. **Detector:** allows measurement of the incoming signal, which is subsequently processed by a computer to determine the intensity. They usually consist of several photomultipliers, each specific for a wavelength range.

Figure 1.13: Fluorometer block diagram [F13]



Also in the case of fluorimetry, as in UV-VIS spectrophotometry, qualitative and quantitative analysis can be carried out.
In the former, the sample is irradiated with different wavelengths and then the emission or excitation spectrum is recorded and compared with databases in order to determine the compound. In quantitative analysis, on the other hand, a specific wavelength is used and a calibration line or curve is created using samples at known concentrations. At this point by measuring the emission intensity, the concentration of the unknown sample can be traced using the following equation:

$$I_t = I_0 * B(I - 10^{-kct})$$

Where:

- I_t is the fluorescent radiation intensity
- I_0 is the incident radiation intensity
- B is the % of incident radiation absorbed
- k is the extinction coefficient
- c is the solution concentration
- t is the solution thickness under test

Compared to classic spectrophotometric techniques, the main advantages of fluorimetry are high sensitivity (even picogram order), high selectivity, and linearity of response over a very wide range of concentrations.

1.6.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC), also known as high-pressure liquid chromatography, is a liquid chromatographic technique for separating two or more compounds present

in a solution.
This technique is based on the co-presence of two phases:

1. **Stationary phase:** consists of a liquid that is supported or chemically bonded on a solid placed inside a glass or steel column packed with materials of fine and controlled grain size (in the order of microns). This allows for increased contact surface area between the mobile and stationary phase and thus more homogeneous packing.

2. **Mobile phase:** these are generally pure solvents or mixtures of two or more solvents that are driven by large pressures through the column with the sample. The solvents must have properties such as high purity, low cost, low viscosity, low toxicity and non-flammability.

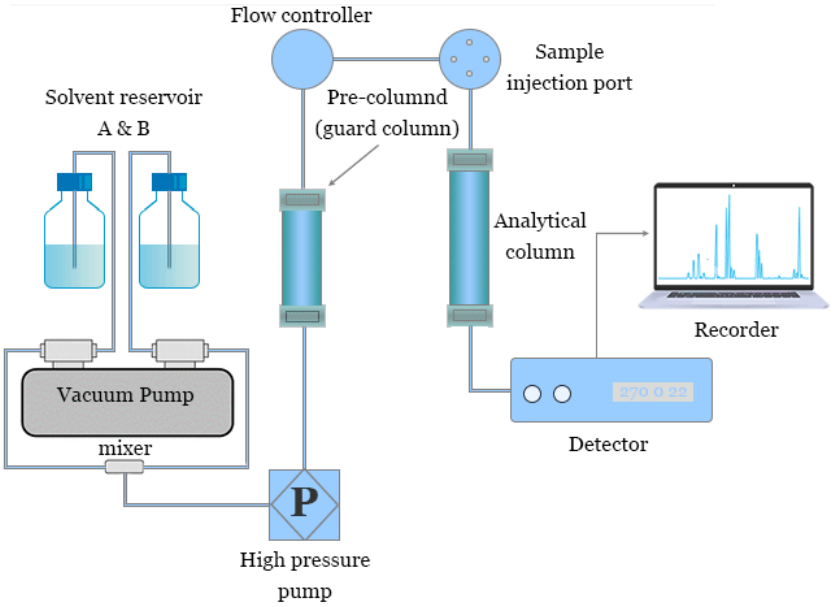
The separation of compounds is generated by their different affinity for the stationary phase placed inside the chromatographic column and the mobile phase flowing through it. Therefore, a substance with a higher affinity for the stationary phase than the mobile phase takes a longer time to flow through the chromatographic column than a substance with a low affinity for the stationary phase and high affinity for the mobile phase. The retention time of a substance, which represents the time it takes for a substance to escape from a column, is influenced by the polarity of the eluent, and therefore the type, intensity and number of interactions between the substance and the two phases. Another variable influencing retention time is the molecular weight of the compounds: for the same number of interactions, compounds with higher molecular weights will take longer to exit the column.
As an output from an HPLC device, a chromatogram, a graph containing so-called chromatographic peaks, is obtained. Each peak represents the outflow of a substance from the column and can be analyzed to determine the features of the substance. The x-axis represents time, while the y-axis represents the intensity of the signal exiting the column as measured by a detector. It is, therefore, possible to determine time-related parameters, such as retention time, or, for example, peak height or area, which provide information about the amount or concentration of the substance.

As reported in Fig. 1.14, the main components of an HPLC apparatus are as follows:

1. **Mobile phase containers:** are one or more containers that contain the solvents used as mobile phase. They can be integrated with degassers, filtration systems and distillers. HPLC separations can be performed with isocratic elution, that is, using an eluent whose composition does not vary during the analysis, or with gradient elution, in which the nature of the eluent varies during the analysis in a continuous or stepwise manner.

2. **High pressure pump:** this device generates the high pressure that allows the mobile phase and sample to flow through the stationary phase. It must therefore be able to generate high pressures (up to hundreds of atmospheres), maintain stable pressure values, deliver precise flows of mobile phase (usually between 0,1 and 10 ml/min), and resist corrosion. The most commonly used pumps are pistons, syringes, and pneumatic pumps.

Figure 1.14: HPLC block diagram [F14]



3. **Sample injection port:** there are several types, automatic and manual. Their function is to inject a well-defined amount of sample, usually varying between 5 and 500 µl depending on the method.

4. **Column:** this is the component that enables the separation of various compounds. They are usually constructed of stainless steel or thick glass, with a length between 10 and 30 cm and internal diameter between 2 and 4.6 mm. They are filled with different types of fill materials (such as microporous silica, alumina, or ion-exchange resin) with particles between 3.5 and 10 µm in diameter. Some columns are also equipped with a thermostatic furnace that can heat and maintain the column at a given temperature. This affects the elution rate of HPLC and allows to obtain better chromatograms.

5. **Detector:** these can be of different types, such as UV absorption, fluorescence (for protein compounds), refraction, electrochemical or mass spectrometer. Regardless of their nature, their goal is to quantify the compound exiting the column, allowing chromatographic peaks to be plotted on the chromatogram.

HPLC is an extensively used separation technique because it has a small column size, which avoids problems with longitudinal deviations and alternate paths, constant and adjustable elution rate, low run time, and the need for small amounts of compound for analysis. This ensures high accuracy and precision.
The main disadvantages associated with this technique are the high cost and the continuous maintenance required to maintain the device.

1.6.5 In-vitro cell analysis

Cell culture is a fundamental biological and biomedical research technique involving growing cells under controlled conditions. Typically these techniques are performed outside their natural environment (in-vitro analysis) but sometimes they are performed directly in the real cellular environment (in-vivo tests) to obtain more reliable data.
They provide a simplified model to study the physiology and biochemistry of cells, the effects of drugs, the mechanisms of diseases and the effects related to material biocompatibility.
Depending on the specific research purposes various cell types can be cultured, such as stem cells, fibroblasts, epithelial cells, and cancer cells. Generally, cell culture systems can be classified into:

- **Primary cultures:** cells are directly derived from tissues. They closely mimic the in vivo state but have some drawbacks such as the limited lifespan.
- **Cell lines:** consist of immortalized cells that can be propagated indefinitely. They offer the advantage of consistent availability and ease of use.

In vitro studies offer several advantages, including high reproducibility, control over experimental conditions, and the possibility to conduct high-throughput screening.
Common in vitro tests include proliferation assays (measure cell growth and replication), migration and invasion assays (assess cell motility) and apoptosis assays.

Several key components are required to create a suitable cell growth and maintenance environment, thus obtaining a successful cell culture. These components include:

1. **Incubator:** it provides an appropriate environment, managing the temperature (usually 37°C for mammalian cells), humidity, and CO_2 concentration (commonly from 5% to 10%).
2. **Sterilization equipment:** several tools are used to maintain a sterile environment and to prevent contamination. Examples include laminar flow hoods, autoclaves, sterilizing filters and ethanol.
3. **Culture vessels:** cells grow in appropriate containers such as flasks, Petri dishes, or multi-well plates. They are usually made of treated plastic or glass, both able to promote cell growth and adhesion.
4. **Culture medium:** it consists in the solution that provides the essential nutrients, growth factors, hormones, and gases required for cell survival and proliferation. Its main components are the basal medium (amino acids, vitamins, inorganic salts, glucose), horse or bovine fetal serum, buffers, and antibiotics.
5. **Cell lines:** consist of the cells being cultured, which can be primary cells or established cell lines (immortalized cells that can proliferate indefinitely).

The main steps to perform during cell culture are critical and fundamental to ensure their viability and proliferation. The most important are:

- **Sterilization and medium preparation:** consists of sterilize all the instruments (culture vessels, media, pipette) and preparing and equilibrating the solutions to the appropriate temperature and pH.
- **Cell seeding:** cells are counted using a hemocytometer or automated cell counter, diluted to the desired concentration and evenly seeded in the culture vessel.
- **Incubation:** culture vessel is placed in the incubator, previously set to the appropriate conditions (temperature, CO_2 concentration, humidity).
- **Monitoring and maintenance:** cells are regularly checked under a microscope to determine confluency, morphology, and possible signs of contamination. The culture medium should be periodically replaced, removing waste products and replenishing nutrients. If cells reach a certain confluency, passaging is required. They are subcultured into a new culture vessel to give them more space for continued growth.

Cytotoxicity Assays

Cytotoxicity has been defined as the capacity of a substance or material to cause damage to cells, leading to their dysfunction or death.
Usually cytotoxicity can manifest as reduced cell viability, impaired cellular function, or induced cell death.
Cytotoxicity assays are biological tests designed to evaluate the potentially harmful effects of substances and materials on cultured cells. All these tests are strictly required during drug development procedures, in environmental toxicology and for safety assessment procedures for chemicals and materials.
It's a fundamental step to develop and refine biomaterials and medical devices, ensuring biocompatibility and safety.
The most used cytotoxicity assays measure the cell metabolic activity (MTT), the amount of released enzymes (LDH), the cell membrane integrity (Trypan Blue Exclusion Test) and the cell size, granularity or specific marker presence (flow cytometry).

Chapter 2

MATERIALS AND METHODS

All the tests and analyses performed in this study (except in vitro tests) were carried out at Tiss'You S.r.l., a company located in Domagnano (RSM) focused on researching and developing biomaterials and medical devices designed to enhance the natural regenerative ability of the human body.
The in vitro tests, instead, were performed in the Hopkins Building belonging to the University of Reading (UoR, Reading, UK). It represents the facility dedicated to pharmaceutical and biomedical sciences.

The collagen used in this study is a Tiss'You S.r.l. product called *"Collygen"*, reported in Fig. 2.1. It is a resorbable membrane consisting of highly purified equine-derived type I atelocollagen. This product is nowadays used for Guided Tissue Regeneration (GTR) and Guided Bone Regeneration (GBR) procedures. Its function is to act as a biological barrier, protecting the bone graft and ensuring optimal protection of the implant site from soft tissue infiltration. At the same time, it allows rapid and effective adhesion to the bone surface without the need for fixation and is completely resorbable in about 4-6 weeks. It thus participates in the in-situ bone healing process by improving tissue quality and its subsequent remodelling.

This elaborate aims to enhance collagen functionality by decorating collagen with RGD, a BP that facilitates cell adhesion and spreading, particularly that of osteoblasts. It also promotes cell proliferation, osteoblast differentiation and mineralization.
Due to all these positive effects related to the presence of RGD, the resulting material would have numerous advantages to the starting one, also opening the door to new potential uses. For example, it could be used as a scaffold in CTE or biomaterial in ATE, and in general in a variety of RM applications, especially those related to the bone compartment.

All the reagents, devices and method used in this study are listed below.

Figure 2.1: "Collygen" membrane produced by Tiss'You S.r.l. [F15]

2.1 REAGENTS

- "VWR Chemicals" Hydrochloric Acid 37% - CAS No.: 7647-01-0
- "Emsure" Acetic Acid 96% - CAS No.: 64-19-7
- "Sigma-Aldrich" Peptide-2000 (Arg-Gly-Asp, RGD) CAS No.: 99896-85-2
- "Sigma-Aldrich" N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) CAS No.: 25952-53-8
- PBS Buffer:
  - "Sigma-Aldrich" Sodium phosphate dibasic CAS No.: 7558-79-4
  - "Sigma-Aldrich" Sodium phosphate monobasic CAS No.: 7558-80-7
  - "Sigma-Aldrich" Sodium chloride CAS No.: 7647-14-5
- "Sigma-Aldrich" 2-Morpholineethanesulfonic acid hydrate (MES Hydrate) CAS No.: 1266615-59-1
- "Sigma-Aldrich" QuantiPro BCA Assay Kit:
  - QuantiPro Buffer QA
  - QuantiPro BCA QB
  - Copper (II) sulfate solution
- "Invitrogen" Qubit Protein Assay Kit:
  - Qubit protein Buffer
  - Qubit protein reagent
  - Qubit protein Standard #1

  - Qubit protein Standard #2
  - Qubit protein Standard #3
- "Alfa Aesar" Guanidine Hydrochloride 99+% CAS No.: 50-01-1
- "Sigma-Aldrich" Sodium hydroxyde 98+% CAS No.: 1310-73-2
- 2-Mercaptorthanol CAS No.: 60-24-2
- "VWR Chemicals" Calcium chloride CAS No.: 10043-140-8
- "Sigma-Aldrich" Proteinase K from Tritirachium album CAS No.: 39450-01-6
- "Uvasol" Trifluoroacetic acid CAS No.: 76-05-1
- "Honeywell" acetonitrile CAS No.: 75-05-8
- "Invitrogen" SDS-PAGE Kit:
  - NuPAGE 4-12
  - PageRuler Plus Prestained Protein Ladder
  - NuPAGE LDS Sample Buffer (4x)
  - NuPAGE Sample Reducing Agent (10x)
  - SYPRO Ruby protein gel stain
  - NuPAGE MES SDS Running Buffer (20x)
- 70% ethanol
- "Gibco" DMEM/F-12 for mammalian cell culture
- "Gibco" StemPro Osteogenesis Differentiation Kit
- "Abcam" Alkaline Phosphatase Staining Kit

2.2 DEVICES

- “Ohaus” Scout portable balance STX1202
- “Ohaus” AdventurerTM Analytical balance AX124
- “Ohaus” frontier 5816r Centrifuge
- “DK SONIC” Ultrasonic cleaner DK-80
- “ELGA Veolia” PURELAB Flex 3 water dispenser and deionization system

- “Ohaus” thermal agitator for incubation and cooling
- "Telstar" LyoBeta Freeze Dryer
- "PerkinElmer" LAMBDA 365 UV/Vis Spectrophotometer
- "Ratiolab" Halbmikro cuvettes UV
- "Invitrogen" Qubit 4 Fluorometer
- "Invitrogen" Qubit Assay Tubes
- "PerkinElmer" Flexar HPLC system
- "NuGen" syringe 3ml + "Rephile" RephiQuik Syringe Filter
- "Brownlee" SPP PeptideES-C18 HPLC column (2.1x150mm, 2.7micrometer)
- “Invitrogen” Mini Gel tank
- “Life Technologies” PowerEase 300W
- “Invitrogen” iBright FL1000 Imaging Systems
- Laminar flow hood
- "Sanyo" Compact 5% CO_2 incubator
- "Grant" unstirred water bath 37°C
- 96-well plate

2.3 METHODS

Starting from the initial material, *“Collygen”* collagen produced by Tiss’You S.r.l., arriving at in vitro cell tests, this study can be divided into four major parts:

1. Starting material characterization
2. Decoration reaction
3. Bond formation verification
4. In vitro tests

In the section below, each of these parts will be analyzed in detail.

2.3.1 Starting Material Characterization

As a first analysis, SDS-PAGE gel electrophoresis was conducted in order to verify and ensure that the starting collagen had the essential characteristics of a top-quality product. This electrophoretic technique allows the determination of the collagen molecular weight, as well as the detection of any dimers/trimers and by-products with lower molecular weights.
A 3mg "Collygen" collagen sample has been solubilized in a solution of acetic acid 0,1 M and incubated overnight at 300 rpm to extract collagen. Then 3 µl of the sample was taken and added with 5 µl of LDS sample buffer, 2 µl of DTT and 10 µl of Milli-Q water, for a total volume of 20 µl. The solution was incubated for 30 min at 500 rpm and then centrifuged at 16000 rpm for 10 min. This step allows the complete denaturation of collagen, as LDS is a denaturing agent and DTT is a reducing agent able to break the disulfide bridges present between cysteines.
Once has been prepared the electrophoresis instrumentation, 10 µl of the sample solution was loaded onto one well of the gel, and molecular weight markers were loaded into a second well. The transformer was started in constant mode (300 W, 200 V and 0,16 mA), and the electrophoretic run took about 30 min. The gel was then soaked twice in 100 ml of a solution of 50% methanol and 7% acetic acid to fix the proteins on the gel.
The gel has been immersed overnight in 100 ml of Syproruby, a fluorescent dye that allows protein staining. The gel was then washed twice with a solution consisting of 100 ml of 10% methanol and 7% acetic acid.
Finally, the acquisition phase was performed with "Invitrogen" iBright FL1000 Imaging Systems in *Fluorescent Mode*, manually adjusting the exposure time to obtain the best possible image.

2.3.2 Decoration Reaction

In this study, three different procedures have been used to try to bind RGD peptide to the *"Collygen"* collagen membrane. In all three procedures, EDC has been used as the crosslinking agent, but in different concentrations and environments.

PROCEDURE 1: EDC, RGD

Regarding this first procedure (P1), two solutions of RGD and EDC both 2 mM were prepared. They were then mixed 1:1 obtaining a solution of RGD and EDC 1 mM. The latter was stirred for 2 h at a temperature of 4°C in order to activate the peptide. 100 µl of the solution was added to each *"Collygen"* collagen sample of about 3 mg to allow it to be decorated. The samples were then incubated at room temperature for about 4 h and then lyophilized under sterile conditions. In order to make the necessary considerations, some *"Collygen"* collagen samples of the same weight were treated with:

- 100 µl of Milli-Q water.
- 100 µl of EDC 1mM.

In order to have an higher amount of data, P1 has been repeated four times, performing four tests defined T1, T2, T3 and T4.

PROCEDURE 2: MES, EDC, RGD

In the second procedure (P2), a solution of MES 40 mM was prepared and then raised to a pH of 2-4 by the addition of hydrochloric acid. EDC was added to the same solution to obtain a final concentration of 2 M. This solution was then mixed 1:1 with RGD 2 mM, resulting in a final concentration of MES 20 mM, EDC 1 M and RGD 1 mM. The solution has been incubated for 10 min at 37°C to activate the peptide. The development of the reaction in an acidic environment, limits the self-assembly of collagen fibres [48], as EDC is extensively used as a crosslinker for collagen itself. Finally, 100 µl of the solution has been added to each *"Collygen"* collagen sample of about 3 mg to allow it to be decorated. Each sample was then incubated overnight at 4°C and subsequently lyophilized.
In order to make the necessary considerations, some *"Collygen"* collagen samples of the same weight were treated with:

- 100 µl of MES 20 mM.
- 100 µl of MES 20 mM and EDC 1 M.

In order to have more data, P2 has been repeated twice, performing two tests defined T5 and T6.

PROCEDURE 3: Acetic Acid, MES, EDC, RGD

Procedure 3 (P3) is similar to P2, except that initially the 3 mg *"Collygen"* collagen samples have been soaked for 2 h in 100 µl of acetic acid 20 mM. This allows the partial solubilization of the collagen resulting in a more favorable environment for RGD decoration. All subsequent steps are similar to procedure 2. Therefore, before freeze-drying, each sample has been incubated overnight in 200 µl of a solution composed of acetic acid 10 mM, MES 10 mM, EDC 0,5 M and RGD 0,5 mM.
Again, in order to make the necessary considerations, some *"Collygen"* collagen samples of the same weight were treated with:

- 200 µl of acetic acid 10 mM.
- 200 µl of acetic acid 10 mM and MES 10 mM.
- 200 µl of acetic acid 10 mM and MES 10 mM and EDC 0,5 M.

Also in this case, to have more data P3 has been repeated twice, performing two tests defined T7 and T8.

The table 2.1 shows all the samples performed and subsequently analyzed in the various tests, with the related identification codes (ID CODE), which provide information about the reaction environment.

Table 2.1: Procedures and reaction environments with their related codes

PROCEDURE [TEST]	**REACTION ENVIRONMENT**	**ID CODE**
P1	Milli-Q water	P1_1
[T1-T2-T3-T4]	EDC 1 mM	P1_2
	EDC 1 mM, RGD 1 mM	P1_3
P2	MES 20 mM	P2_1
[T5-T6]	MES 20 mM, EDC 1 M	P2_2
	MES 20 mM, EDC 1 M, RGD 1 mM	P2_3
P3	Acetic A. 10 mM	P3_1
[T7-T8]	Acetic A. 10 mM, MES 10 mM	P3_2
	Acetic A. 10 mM, MES 10 mM, EDC 0,5 M	P3_3
	Acetic A. 10 mM, MES 10 mM, EDC 0,5 M, RGD 0,5 mM	P3_4

2.3.3 Bond Formation Verification

Three different qualitative and quantitative techniques have been performed to verify the bond formation between collagen and RGD: UV-VIS spectrophotometry, fluorimetry and HPLC. Before each quantification, samples were washed with 1 ml of PBS 10 mM to solubilise any RGD residues that weren't bound but simply embedded in the three-dimensional collagen structure.

UV-VIS

Through UV-VIS spectrophotometry, proteins and peptides of various sizes can be quantified. By knowing the amount of RGD added in each sample and being able to quantify the RGD present in the washing PBS, by difference, it is possible to trace the amount of RGD bound to the *"Collygen"* collagen membrane.

$$RGD_{bounded} = RGD_{added} - RGD_{PBS}$$

To determine the amount of RGD present in the washing PBS, the Sigma-Aldrich QuantiPro BCA Assay Kit was used. It is a method that allows the quantification of proteins and peptides by bicinchoninic acid (BCA) and has a linearity range between 0,2- 50 µg/ml. The washing PBS was mixed 1:1 with a solution consisting of the three reagents in the kit (QA, QB, QC), for a

total volume equal to 1,5 ml. This solution has been incubated for 1 h at 60°C and then brought back to room temperature. During this step, the proteins and peptides (in this case the RGD peptide) reduce the cupric ions to cuprous in a basic environment, which react with BCA to form a purple complex.
Next, once the calibration line has been created through three points (0;5;30 µg/ml), the absorbance of each sample was measured at a wavelength of 562 nm with PerkinElmer LAMBDA 365 Spectrophotometer. This allow to determine the concentration of the RGD solution and therefore the quantification of RGD (in µg) present in the washing PBS. Then, with the above-mentioned formula, has been possible to determine the amount of RGD covalently bound to the collagen membrane.

FLUORIMETRY

To quantify proteins and peptides of various sizes it's possible to use also a fluorimetric method. As reported in the paragraph above, determining the amount of RGD present in the washing PBS and knowing the RGD added quantity, by difference, it is possible to trace the amount of RGD bound to the *"Collygen"* collagen membrane.
To determine the amount of RGD present in the washing PBS, the Qubit Protein Assay Kit has been used. It is a method that allows the quantification of proteins and peptides in a wide range of concentrations, from 12,5 µg/ml up to 5 mg/ml. The washing PBS was mixed 1:10 with the previous diluited Working Solution present in the kit, for a total volume equal to 200 µl.
The calibration line was created by using the three standards provided by the kit (#1, #2, #3) and the fluorescence of each sample was measured with the Invitrogen Qubit 4 Fluorometer. This allow to determine the concentration of the RGD solution and therefore the quantification of RGD (in µg) present in the washing PBS.

HPLC

HPLC represents a key analytical method for conducting qualitative and quantitative assessments on the *"Collygen"* collagen membrane, both before and after the decorating process, in order to detect any changes in molecular weight and concentration. For this reason, one sample for each procedure (for a total of 10 samples) has been analysed with this technique.
Before proceeding with the analysis by HPLC, it is essential to denature and then digest the collagen sample. For this purpose, a solution containing guanidine-HCl 6 M, PBS 50 mM and β-mercaptoethanol 2 mM was prepared and brought to a pH 8 by sodium hydroxide. 300 µl of this solution have been added to each collagen sample, followed by incubation at 60°C for 1 h. This denaturation process allows the protein to be arranged, unwinding the collagen chains and breaking the disulfide bridges between cysteines. Then, the solution was cooled and diluted with 600 µl of PBS 50 mM and $CaCl_2$ 5 mM to obtain a final guanidine-HCl concentration equal to or less than 2 M.
The next step involves the digestion of collagen. In this context, a K proteinase concentration of

50 µg/ml has been added to each sample. Incubation was performed at 55°C overnight. K Proteinase acts preferentially on protein cleavage near hydrophobic amino acid residues (aliphatic, aromatic or other) and has a molecular mass of 28,9 kDa. After digestion, the samples were filtered by a 0.22 µm syringe filter, to remove any impurities or parts of collagen that were not fully digested.
HPLC analyses have been conducted using the PerkinElmer Flexar HPLC system. Operating parameters included a flow rate of 0,5 ml/min, an injection volume of 20 µl for each sample, a temperature of 55°C, absorbance detection at 220 nm, and a solvent gradient transition. The two solvents used were 0.1% TFA (solvent A) and acetonitrile (solvent B). The total elution time for each sample has been fixed at 26 minutes: from 0 to 25 minutes, the gradient varies from 15% to 65% of solvent B, and then in the last minute from 65% to 95% of solvent B.
For each sample, the chromatogram was recorded, which was then analyzed and interpreted to identify any differences between RGD-treated and untreated collagen samples.

2.3.4 In Vitro Tests

In this part of the study conducted at the University of Reading (UoR, Reading, UK), the following procedures were analyzed: P1_1, P1_2, P1_3, P2_2, P2_3, P3_3, P3_4.
In vitro tests were used to verify whether the presence of RGD enhances collagen osteogenicity, cell adhesion, and cell proliferation. For this purpose, the behaviour of Mesenchymal Stem Cells (MSCs) was analysed.
For each of the above-mentioned procedures, 6 samples weighing approximately 1 mg have been prepared. All the samples have been placed in a 96-well plate, immersed in 100 µl of Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C and 5% CO_2. After three days, around 7000 MSCs and 100 µl of fresh DMEM were added to each well. Subsequently, for each procedure:

- 3 samples remained in the DMEM, refreshed every two days
- 3 samples were cultured in the "StemPro Osteogenesis Differentiation kit" to promote the osteogenic differentiation of MSCs

After 21 days, each sample underwent staining with the ALP staining kit, which measures the alkaline phosphatase activity. The ALP is an enzyme produced by osteoblasts and used to deposit calcium phosphates on the ECM. The ALP hydrolyses 5-Bromo-4-chloro-3-indolyl phosphate group (contained in the kit) to form a blue-coloured intermediate then oxidized producing an insoluble, dark blue dimer, clearly visible with the microscope. Each sample was washed with 100 µl of PBS. After removal, 50 µl of the prepared "Abcam" ALP staining kit was added in each well. The samples were incubated for 30 minutes at 37°C in a 5% CO_2 incubator and then analysed under a microscope. Once acquired the images, they were processed following these steps:

- Original images (RGB) were loaded onto the Fiji software

- Images were converted into greyscale images
- Images were inverted and the background subtracted
- Average pixel intensity was measured and the data were copied into GraphPad Prism software
- Statistical significance was tested using one way ANOVA with Bonferroni post-hoc test.

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Chapter 3

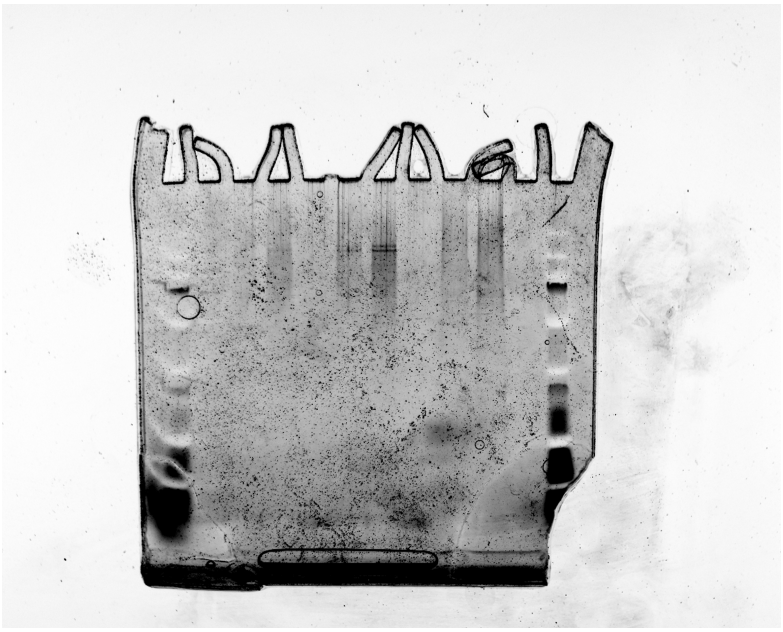
RESULTS

In this section are reported the results obtained from the various analyses carried out at the Tiss'You S.r.l. laboratory and at the University of Reading (UK).

3.1 SDS-PAGE Results

Firstly, in Fig. 3.1 the SDS-PAGE result of the "Collygen" membrane has been reported in order to characterize the starting material. The first and last wells contain the molecular weight markers, instead the central wells contain the "Collygen" samples at two different concentrations.

Figure 3.1: SDS-PAGE result



3.2 UV-VIS and Fluorimetry Results

The UV-VIS spectrophotometry and fluorimetry results have been reported to quantify the amount of unbounded RGD, which corresponds to the RGD solubilized in the washing PBS.

The Table 3.1 shows the four different tests (T1, T2, T3, T4) performed following the Procedure 1 (P1). The table reports each mass sample and the results (in µg of RGD and µg of RGD per mg of collagen) obtained with the spectrophotometric and fluorometric methods.
Tables 3.2 and 3.3 report the amount of added, unbounded and bounded (also %) RGD regarding T1, T2, T3 and T4. To quantify the unbounded RGD, have been compared the method P1_2 and P1_3, because their only difference is the presence of RGD. Thus subtracting the value of P1_3 with P1_2 has been determined the "Unbounded RGD".

The Table 3.4 shows the two different tests (T5, T6) performed following the Procedure 2 (P2). The table reports each mass sample and the results (in µg of RGD and µg of RGD per mg of collagen) obtained with the spectrophotometer and fluorometer.
Tables 3.5 and 3.6 report the amount of added, unbounded and bounded (also %) RGD regarding T5 and T6. To quantify the unbounded RGD, have been compared the method P2_2 and P2_3, because also in this case their only difference is the RGD presence. Subtracting the value of P2_3 with P2_2 has been determined the "Unbounded RGD".

The Table 3.7 shows the two different tests (T7, T8) performed following the Procedure 3 (P3). The table reports each mass sample and the results (in µg of RGD and µg of RGD per mg of collagen) obtained with the spectrophotometer and fluorometer.
Tables 3.8 and 3.9 report the amount of added, unbounded and bounded (also %) RGD regarding T7 and T8. To quantify the unbounded RGD, have been compared the method P3_3 and P3_4, because also in this case their only difference is the RGD presence. Subtracting the value of P3_4 with P3_3 has been determined the "Unbounded RGD".

All the tests weren't considered valid if the "Unbounded RGD" value was negative or greater than the "Added RGD".

3.3 HPLC Results

HPLC technique was performed to evaluate changes in collagen peptides MW after proteinase K hydrolysis, in order to verify the new chemical bond between collagen and RGD. The chromatograms report on the x-axis the elution time (proportional to the collagen peptide MW) and on the y-axis the absorbance (an indicator of the concentration at a given elution time).
Figures from Fig. 3.2 to Fig. 3.11 detail the chromatograms for each sample, identified by their respective procedure code. Fig. 3.12 compares P1_2 and P1_3, highlighting the impact of RGD presence. Similarly, Fig. 3.13 compares P2_2 and P2_3, focusing on the influence of RGD.

Finally, Fig. 3.14 illustrates the comparison between P3_3 and P3_4, emphasizing the sole difference in RGD presence.

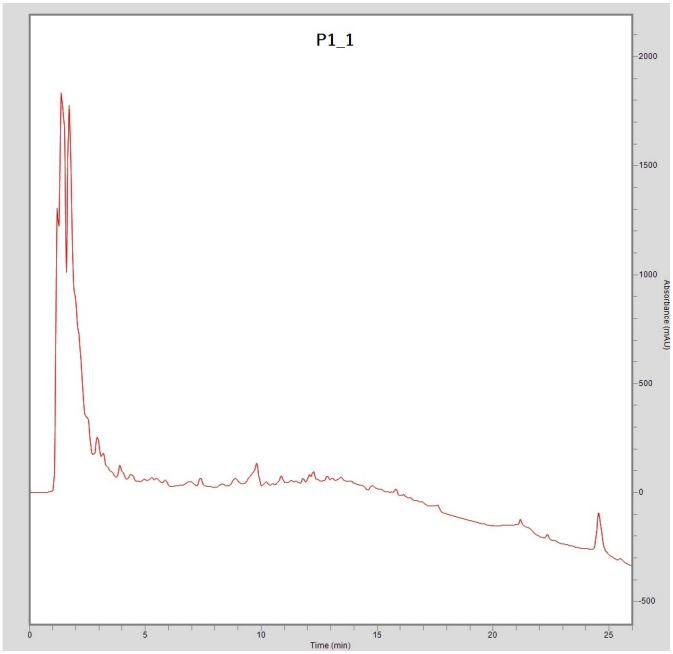


Figure 3.2: HPLC P1_1 result

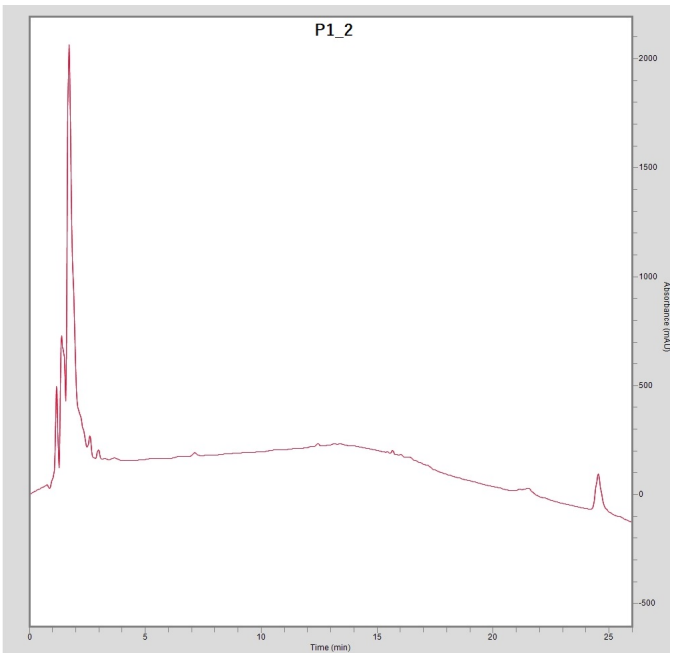


Figure 3.3: HPLC P1_2 result

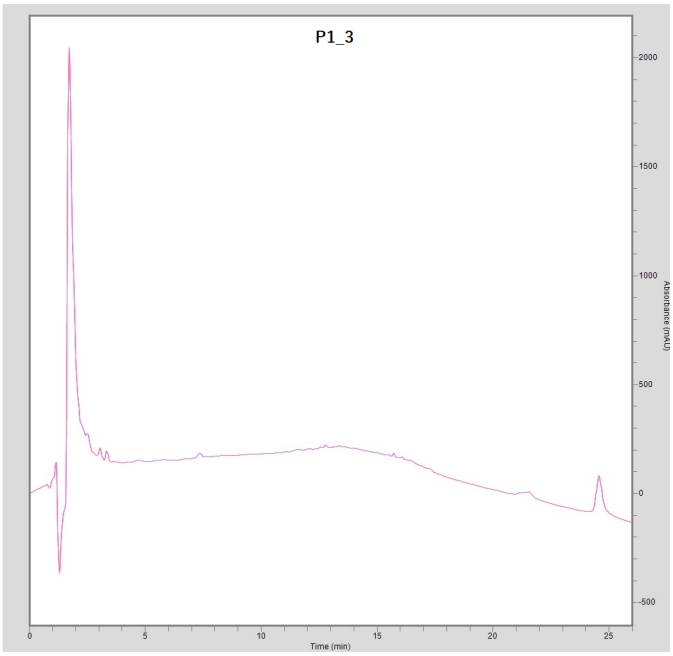


Figure 3.4: HPLC P1_3 result

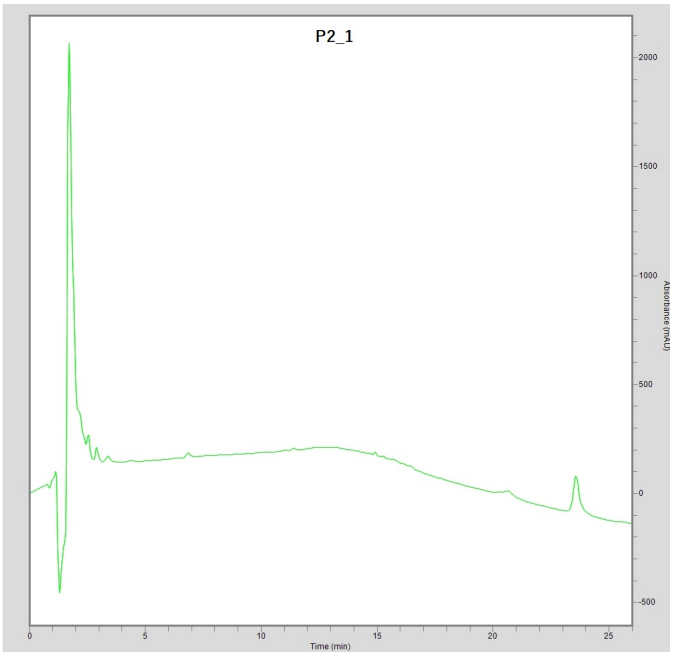


Figure 3.5: HPLC P2_1 result

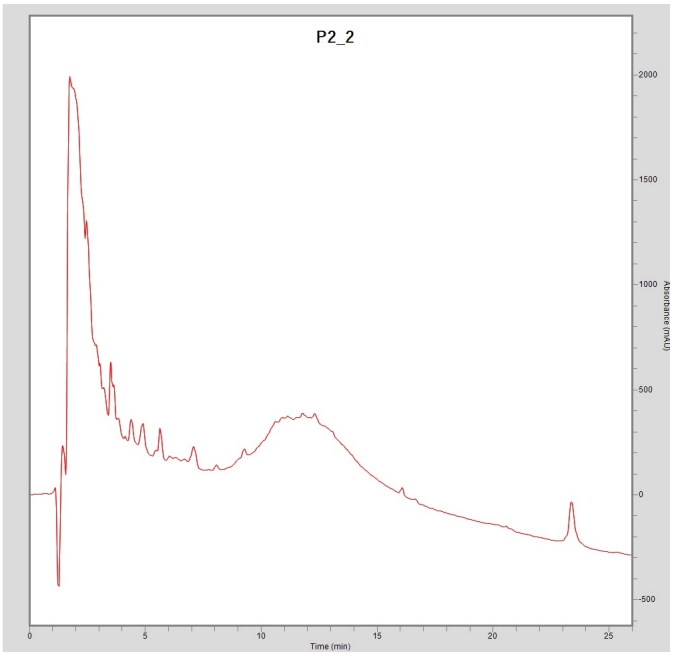


Figure 3.6: HPLC P2_2 result

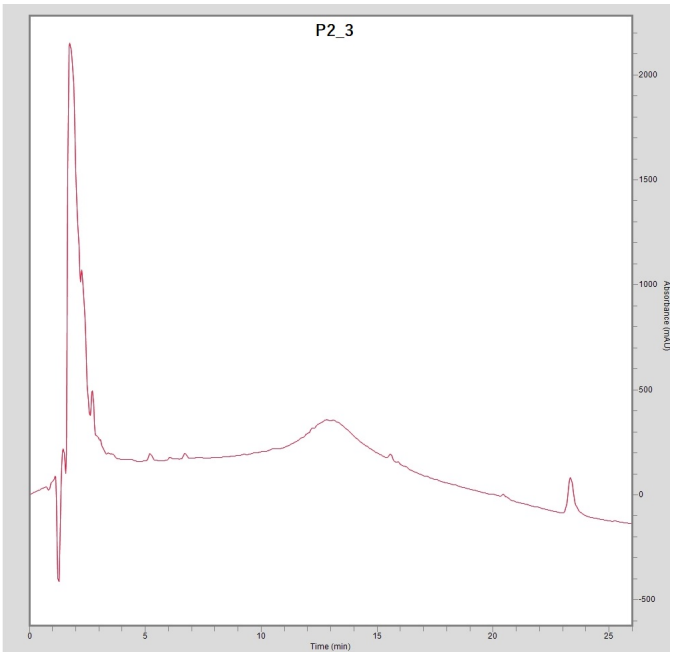


Figure 3.7: HPLC P2_3 result

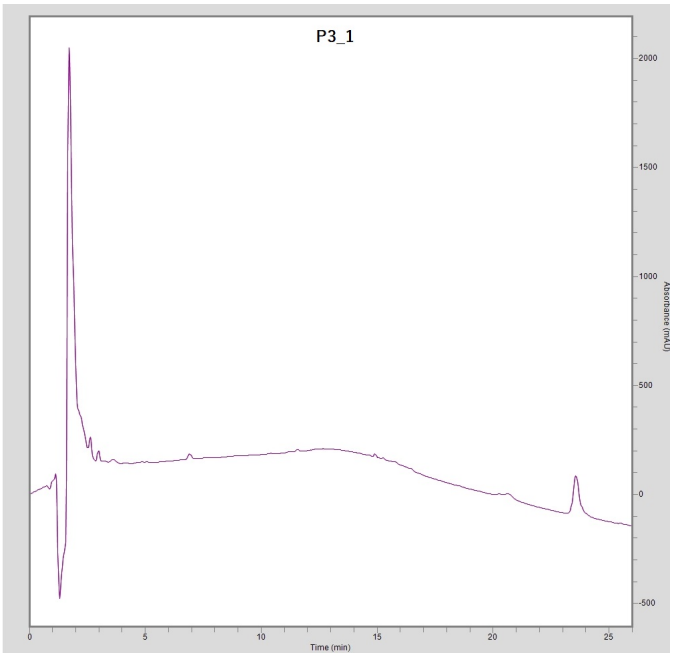


Figure 3.8: HPLC P3_1 result

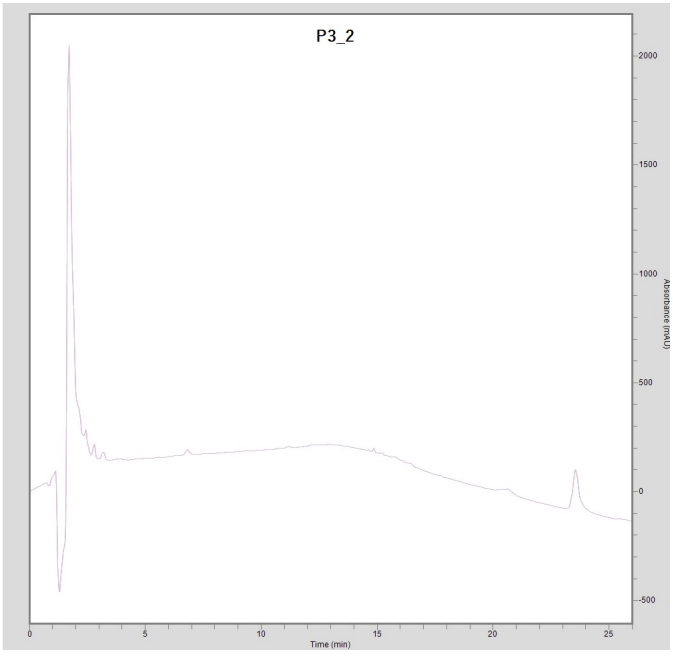


Figure 3.9: HPLC P3_2 result

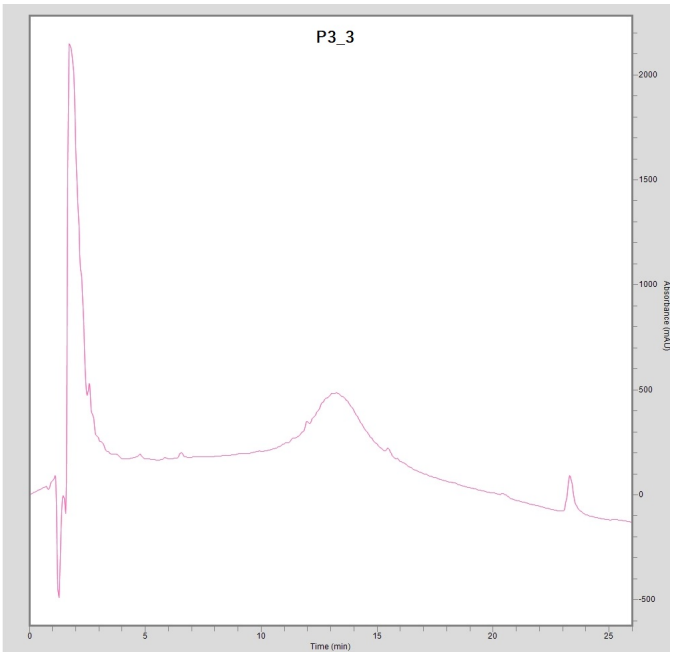


Figure 3.10: HPLC P3_3 result

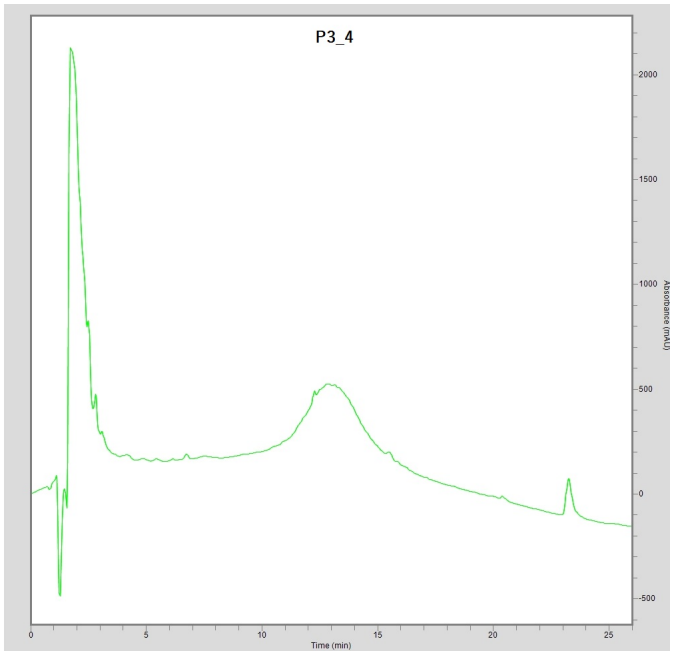


Figure 3.11: HPLC P3_4 result

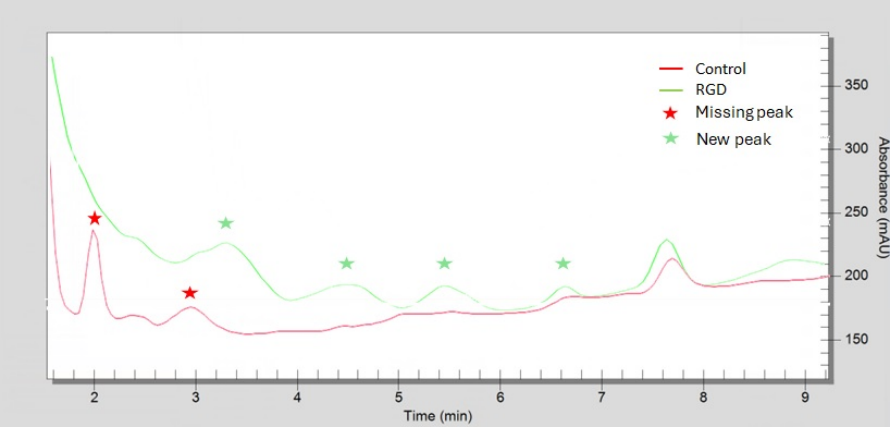


Figure 3.12: Comparison between P1_2 and P1_3

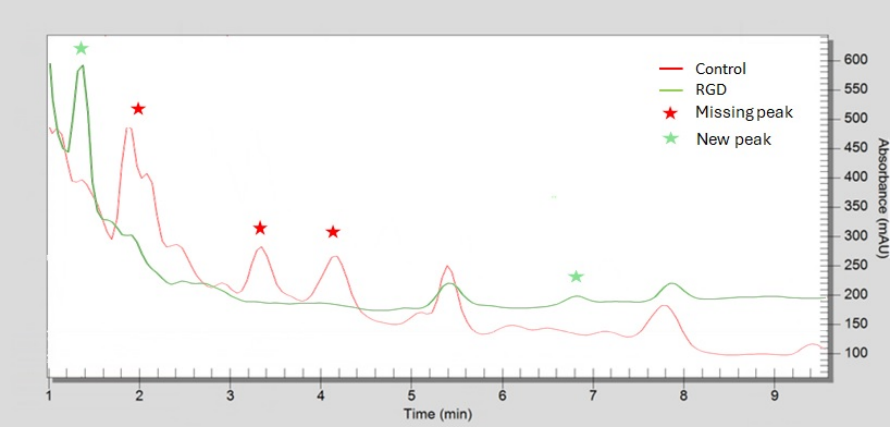


Figure 3.13: Comparison between P2_2 and P2_3

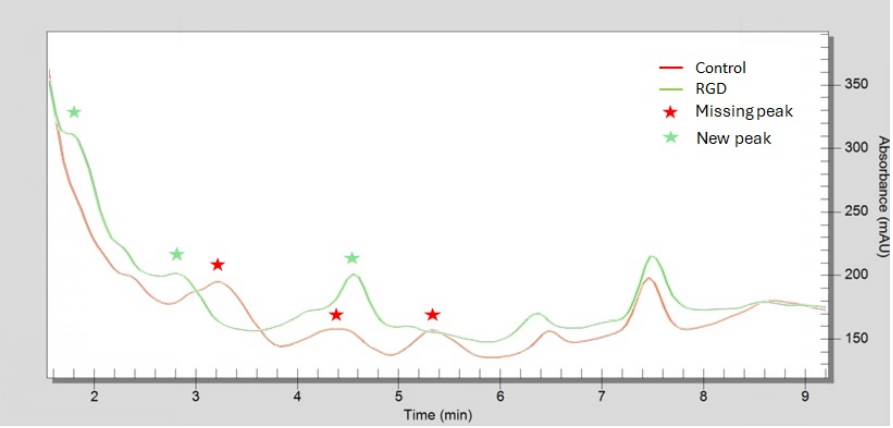


Figure 3.14: Comparison between P3_3 and P3_4

3.4 In Vitro Test Results

In vitro tests have been performed to determine if the RGD presence promotes cell adhesion and collagen osteogenicity. For this purpose, samples were cultured with MSCs in standard DMEM media and osteogenic media. Fig. 3.15, Fig. 3.16, and Fig. 3.17 report the in vitro test results for each sample with the related procedure code obtained with ALP staining 21 days after MSCs seeding in standard DMEM media. In these processed images, the ALP activity is marked with a darker colour.

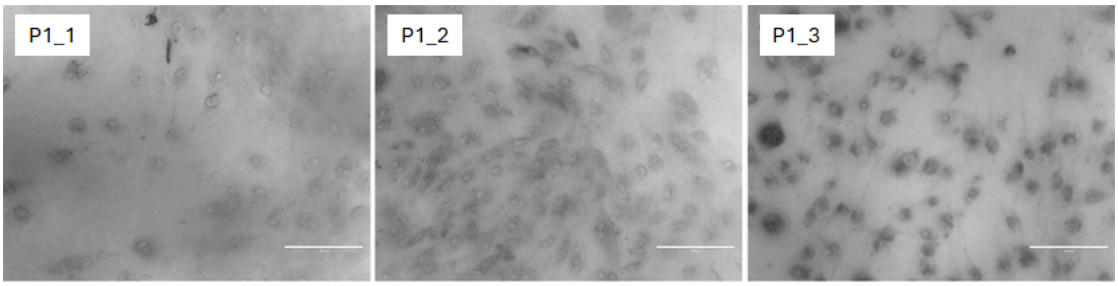


Figure 3.15: ALP activity comparison between P1_1, P1_2 and P1_3

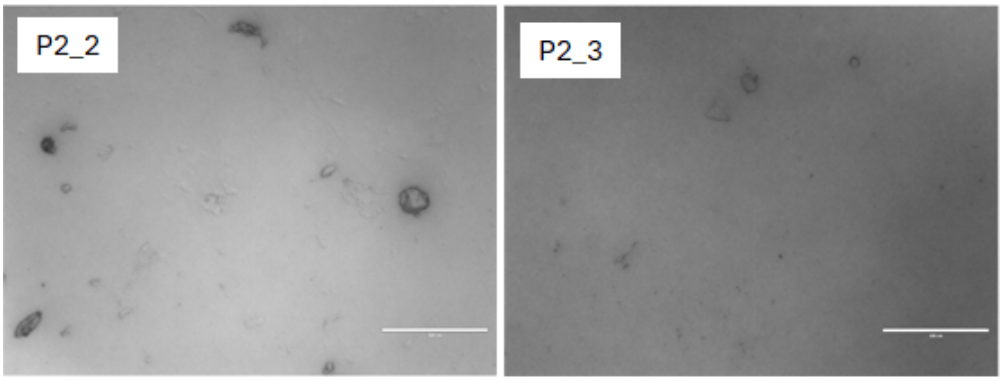


Figure 3.16: ALP activity comparison between P2_2 and P2_3

Fig. 3.18 reports the comparison between P1_1, P1_2 and P1_3 (both in DMEM and osteogenic medium) analyzing the pixel intensity, which is proportional to the ALP activity of the MSCs. In standard DMEM media, ALP activity showed an increment of 46.7%, whereas in osteogenic media, the increment was 81.5%.

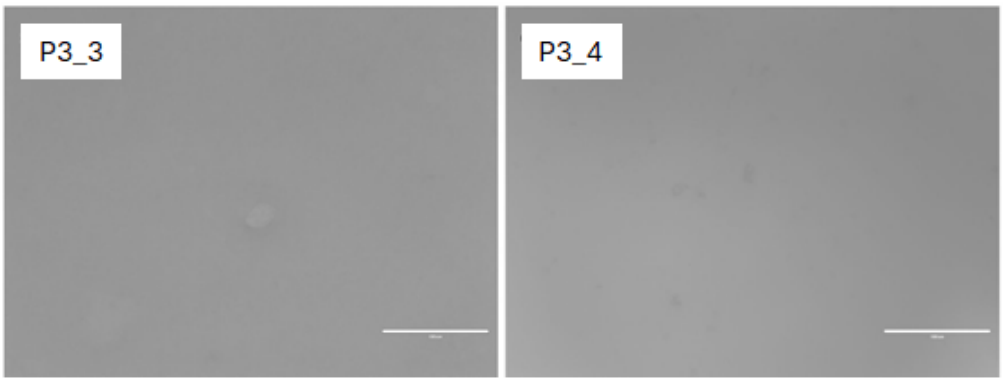


Figure 3.17: ALP activity comparison between P3_3 and P3_4

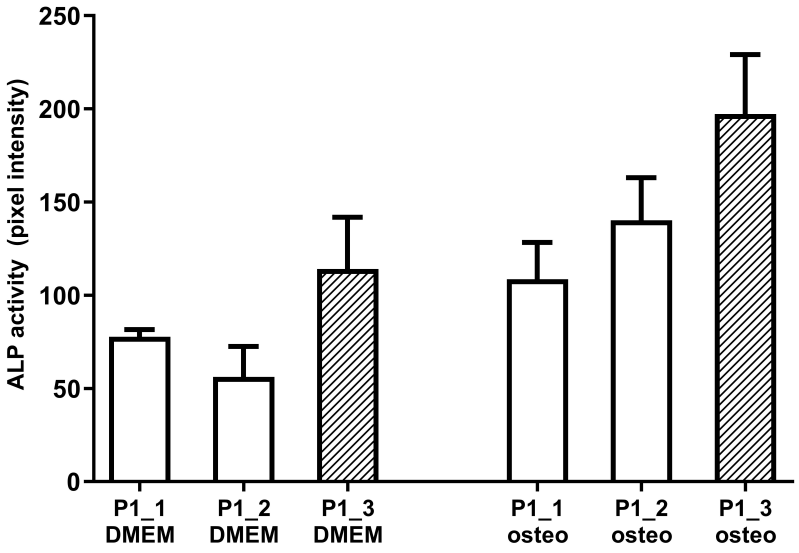


Figure 3.18: ALP activity measured through pixel intensity determination

Table 3.1: P1 results

Test Number	ID Code	Sample Mass (mg)	UV-VIS (µg/ml=µg)	UV-VIS (µg/mg)	FLUOR (µg/ml=µg)	FLUOR (µg/mg)
T1	P1_2	2.4	34.8	14.5	35.0	14.6
	P1_3	2.4	52.8	22.0	59.7	24.9
T2	P1_2	2.0	26.2	13.1	31.4	15.7
	P1_3	2.0	58.3	29.2	63.1	31.6
T3	P1_2	2.1	31.8	15.1	33.0	15.7
	P1_3	2.1	65.5	31.2	60.9	29.0
T4	P1_2	2.3	110.0	47.8	36.8	16.0
	P1_3	2.3	73.7	32.0	64.6	28.1

Table 3.2: UV-VIS P1 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T1	34.6	18.0	16.6	48.0	YES
T2	34.6	32.1	2.5	7.3	YES
T3	34.6	33.7	0.9	2.7	YES
T4	34.6	-36.3	//	//	NO
AVERAGE VALUES	**34.6**	**27.9**	**6.7**	**19.3**	

Table 3.3: Fluorimetry P1 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T1	34.6	24.7	9.9	28.7	YES
T2	34.6	30.0	4.6	13.4	YES
T3	34.6	27.9	6.7	19.4	YES
T4	34.6	27.8	6.8	19.7	YES
AVERAGE VALUES	**34.6**	**27.6**	**7.0**	**20.3**	

Table 3.4: P2 results

Test Number	ID Code	Sample Mass (mg)	UV-VIS (µg/ml=µg)	UV-VIS (µg/mg)	FLUOR (µg/ml=µg)	FLUOR (µg/mg)
T5	P2_2	3.3	245.1	74.3	163.7	49.6
	P2_3	3.3	307.5	93.2	187.8	56.9
T6	P2_2	3.4	226.2	66.5	168.6	49.6
	P2_3	3.4	281.5	82.8	179.9	52.9

Table 3.5: UV-VIS P2 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T5	34.6	62.4	//	//	NO
T6	34.6	55.3	//	//	NO
AVERAGE VALUES	**34.6**	**58.9**	//	//	

Table 3.6: Fluorimetry P2 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T5	34.6	24.1	10.5	30.4	YES
T6	34.6	11.3	23.3	67.4	YES
AVERAGE VALUES	**34.6**	**17.7**	**16.9**	**48.9**	

Table 3.7: P3 results

Test Number	ID Code	Sample Mass (mg)	UV-VIS (µg/ml=µg)	UV-VIS (µg/mg)	FLUOR (µg/ml=µg)	FLUOR (µg/mg)
T7	P3_3	3.2	217.1	67.8	133.5	41.7
	P3_4	3.2	237.2	74.1	162.7	50.8
T8	P3_3	3.2	220.1	68.8	136.2	42.6
	P3_4	3.2	233.9	73.1	169.3	52.9

Table 3.8: UV-VIS P3 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T7	34.6	20.1	14.5	42.0	YES
T8	34.6	13.8	20.8	60.2	YES
AVERAGE VALUES	**34.6**	**17.0**	**17.7**	**51.1**	

Table 3.9: Fluorimetry P3 results

Test Number	Added RGD (µg)	Unbounded RGD (µg)	Bounded RGD (µg)	Bounded RGD (%)	Test Validity
T7	34.6	29.2	5.4	15.7	YES
T8	34.6	33.1	1.5	4.4	YES
AVERAGE VALUES	**34.6**	**31.2**	**3.5**	**10.1**	

Chapter 4

DISCUSSION

In this section will be analyzed all the results obtained at the Tiss'You S.r.l. laboratory and at the University of Reading (UK).

The SDS-PAGE technique has been performed in order to have an initial characterisation of the collagen, determining its MW and the eventual presence of byproducts at lower MW. As it's possible to analyse from Fig. 3.1, the gel presents two defined and strong bands. The upper one corresponds to a MW of 120 kDa, instead the lower one is located at approximately 110 kDa. They perfectly represent the double band typical of type I collagen, formed by $\alpha 1$ chain and $\alpha 2$ chain. Under than this double band there aren't other bands at a lower MW, meaning that the starting "Collygen" collagen membrane has the essential characteristic of a top-quality product and so it's suitable for the following experiments.

For what concern the UV-VIS spectrophotometry and fluorimetry, they have been performed to determine the amount of unbounded RGD solubilized in the washing PBS. Thus, knowing the amount of added RGD and determining the unbounded one, by difference has been possible to quantify the bounded RGD and the consequent reaction yield. All the results concerning these two techniques have been reported from Tab. 3.1 to Tab. 3.9.
Analyzing Tab. 3.2 and Tab. 3.3, it's possible to affirm that using P1 RGD has been successfully linked to collagen, with an average reaction yield of 19.3% (UV-VIS) and 20.3% (fluorimetry).
Examining Tab. 3.6, it's possible to assert that using P2 RGD has been successfully linked to collagen, with an average reaction yield of 48.9% (fluorimetry), higher than results obtained following P1. On the other hand, the results coming from Tab. 3.5 cannot be used for the reaction yield determination, due to an incorrect quantification of RGD. In fact, the amount of unbounded RGD emerges higher than the added RGD. This obvious incorrect data can be related to the peptide release by the collagen membrane, thus during quantification with UV-VIS spectrophotometry, the device quantified not only the unbounded RGD but also the peptides

released by the collagen membrane.
Finally, evaluating Tab. 3.8 and Tab. 3.9 it's possible to affirm that also P3 can chemically bond RGD and collagen. In this case, the average reaction yield corresponds to 51.1% (UV-VIS) and 10.1% (fluorimetry). These two results are quite different from each other in percentage terms, but both confirm the presence of a link between RGD and collagen.
Thus, summarizing the results obtained from UV-VIS spectrophotometry and fluorimetry, excluding P2 with UV-VIS, it is possible to assess the successful binding between the RGD peptide and collagen.

Analyzing the HPLC results, can be noticed different conditions able to influence the reaction between RGD and collagen.
To obtain relevant data regarding P1 the comparison between Fig. 3.3 and Fig. 3.4 is highlighted in Fig. 3.12. It can be observed that 2 peaks disappear and 4 new peaks appear after RGD decoration at a higher retention time. This confirms that using P1 the collagen increases in its MW thus the RGD is successfully linked to the collagen membrane.
To obtain relevant data regarding P2 the comparison between Fig. 3.6 and Fig. 3.7 is highlighted in Fig. 3.13. It can be noticed that 3 peaks disappear and 2 new peaks appear after RGD decoration and the green baseline (RGD decorated collagen) is higher than the red one (control collagen). This confirms that using P2 the collagen MW increases thus the RGD has been successfully linked to the collagen membrane.
Finally, to obtain relevant data regarding P3 the comparison between Fig. 3.10 and Fig. 3.11 is highlighted in Fig. 3.14. It can be observed that 3 peaks disappear and 3 new peaks appear after RGD decoration, and also in this case the green baseline (RGD decorated collagen) is higher than the red one (control collagen). This confirms that also using P3 the collagen MW increases thus the RGD was successfully linked to the collagen scaffold.
Evaluating the complete chromatograms of all the procedures and considering the collagen digestion time with protease K, it can be affirmed that:

- **EDC crosslinks collagen**: chromatograms related to samples treated with EDC (Fig. 3.3, Fig. 3.4, Fig. 3.6, Fig. 3.7, Fig. 3.10, Fig. 3.11) show different pattern respect to samples untreated with EDC (Fig. 3.2, Fig. 3.5, Fig. 3.8, Fig. 3.9). Some peaks disappear, there is a different trend and the coelution band presents a higher and larger peak. These affirm not only that the RGD is linked to the collagen but also that EDC crosslinks the collagen fibres, obtaining new peptides after K proteinase hydrolysis. In fact, especially samples treated with high concentration EDC (Fig. 3.6, Fig. 3.7, Fig. 3.10, Fig. 3.11), show these effects more than the others. The collagen crosslinking is also confirmed by the fact that samples treated with high-concentration EDC had a longer digestion time (1 day) than samples untreated with EDC (30 minutes).
- **Acetic Acid presence isn't determinant for coupling reaction**: the acetic acid should provide a slight increase in the solubilization of collagen fibres, determining a bet-

ter result and reaction yield. However the HPLC results, combined with UV-VIS and fluorimetry results, don't confirm that.

- **MES presence isn't determinant for coupling reaction**: as for the acetic acid, the MES presence should improve the reaction yield, being the MES a surfactant able to unroll the collagen chains. Also in this case, it probably doesn't influence the HPLC results, thus it's possible to affirm that is not essential for the coupling reaction between RGD and collagen.

Thus, evaluating all the results obtained with HPLC, it's possible to confirm that both of P1, P2, and P3 can be used to decorate collagen with RGD peptide. Moreover, high concentrations of EDC also induce collagen crosslinking.

Analyzing the in vitro results obtained from ALP staining 21 days after MSCs seeding, it is evident that the different procedures yield distinct outcomes.
In Fig. 3.15, the first image represents the "Collygen" collagen scaffold, used as the control condition (P1_1). The untreated collagen is obviously non-cytotoxic, allowing MSCs to adhere and proliferate without issues. It is important to note that collagen is slightly osteogenic per se, thus some MSCs begin to differentiate into osteoblasts even without RGD decoration. The second image depicts P1_2, where the collagen scaffold was treated with 1 mM EDC. This treatment slightly enhances cell adhesion and proliferation, although ALP activity is lower compared to the control collagen. The third image shows P1_3, where the collagen scaffold was treated with both 1 mM EDC and 1 mM RGD. This treatment significantly promotes cell adhesion, cell proliferation, and ALP activity, indicating that the presence of RGD markedly enhances the differentiation of MSCs into osteoblasts.
Fig. 3.16 presents the results for P2_2 and P2_3. These procedures decrease cell adhesion and proliferation compared to the control collagen (P1_1), and the environment appears to be slightly cytotoxic. This phenomenon can be attributed to the high concentration of EDC (1 M) and the presence of MES, which acts as a surfactant and can inhibit the cell attachment to the collagen scaffold.
Finally, Fig. 3.17 illustrates the results for P3_3 and P3_4. The absence of cells in these conditions indicates cytotoxicity. The underlying causes are similar to those in the previous section, exacerbated by the presence of acetic acid, which lowers the pH and creates an environment unsuitable for MSC viability.
The processed image results, presented in Fig. 3.18, confirm that P1_3 is the most effective procedure concerning ALP activity. Furthermore, the presence of osteogenic media during MSC proliferation further enhances osteoblast differentiation, highlighting the positive effect of RGD. Infact in P1_3 the pixel intensity which is related to ALP activity is 46.7% higher than in P1_1 (control collagen) in DMEM media and 81.5% higher in osteogenic media.

Chapter 5

CONCLUSIONS

This study aimed to enhance the functionality of a collagen scaffold by decorating it with a bioactive peptide, RGD, capable of increasing cell adhesion, cell proliferation, and collagen osteogenicity. This work is a pilot study for a larger project aimed at decorating collagen scaffolds with different bioactive peptides to achieve the so-called cellular programming, the ability to obtain desired actions at tissue and cell levels.

In this study, three different procedures were used to chemically bind RGD to collagen, all through the use of EDC, a carbodiimide, as a crosslinker:

- **P1**: Collagen, EDC 1 mM, RGD 1 mM
- **P2**: Collagen, MES 20 mM, EDC 1 M, RGD 1 mM
- **P3**: Collagen, Acetic Acid 10 mM, MES 10 mM, EDC 0,5 M, RGD 0,5 mM

After the reaction, UV-VIS spectrophotometry, fluorimetry, and HPLC were used to verify the RGD decoration and to determine the reaction yield. Summarizing all the results obtained with these three techniques, it is possible to confirm that all three procedures (P1, P2, P3) can be used to positively decorate collagen with RGD. Additionally, procedures P2 and P3 have the highest reaction yields. and high concentrations of EDC cause significant crosslinking of collagen, considerably increasing the collagen digestion times with protease K (from 30 minutes to about one day).

However, the results obtained from the in vitro tests conducted at the University of Reading (UK) demonstrate that procedures P2 and P3, despite the higher reaction yield, are cytotoxic to MSCs. This cytotoxicity can be related to the high concentration of EDC (1 M in P2 and 0,5 M in P3), the use of MES (a surfactant which can reduce the cells' ability to adhere to the collagen scaffold) and to the presence of acetic acid (which lowers the pH, creating an environment

unfavourable for cell viability).
In contrast to these results, procedure P1, which involves treating the collagen scaffold with both 1 mM EDC and 1 mM RGD, yielded excellent results, as cell adhesion and cell proliferation were visibly increased. Furthermore, with ALP staining, which determines the activity associated with the differentiation of MSCs into osteoblasts, it was possible to confirm that collagen decorated with RGD following P1 induces osteogenesis more effectively than untreated collagen.

Therefore, the objective of this work has been achieved, determining that procedure P1 is the best in terms of cell adhesion and proliferation, with a reaction yield of approximately 20%. Additionally, the presence of RGD genuinely increases the osteogenicity of collagen, increasing the ALP activity of 46.7% (in DMEM media) and 81.5% (in osteogenic media) if compared with undecorated collagen.

Chapter 6

FUTURE WORKS

As previously mentioned, this work is a pilot study that is part of a larger project aimed at enhancing the functionality of collagen with various types of bioactive peptides to achieve cellular programming.
In this specific case, the collagen scaffold was decorated with RGD, a bioactive peptide capable of enhancing cell adhesion, cell proliferation, and the osteogenic potential of collagen. It has been demonstrated that the presence of RGD increases ALP activity in cells by 46.7% when cultured in standard medium (DMEM) and by 81.5% when cultured in osteogenic medium (StemPro).

The next steps involve decorating the collagen scaffold with other types of bioactive peptides capable of promoting the differentiation of MSCs not into osteoblasts but, for example, into fibroblasts, endothelial cells, etc.
The process to be followed remains the same: identify the bioactive peptide capable of performing a specific cellular task, find the appropriate method to bind it to the collagen scaffold, and finally conduct in vitro tests to verify the successful achievement of the task.
Only at that point can it be asserted the full achievement of cellular programming.

Chapter 7

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Bibliography

[1] "Renewed Interest in Regenerative Medicine" *EBioMedicine* 2018 Jan;27:1-2. doi: 10.1016 .ebiom.2018.01.009. PMID: 29413181; PMCID: PMC5828542.

[2] Goodman SR. "Introduction to the Regenerative Medicine Thematic Issue" *Exp Biol Med (Maywood)* 2021 Aug;246(16):1789-1790. doi: 10.1177/15353702211029754. PMID: 34412528; PMCID: PMC8381700.

[3] Hussain Y, Khan H. "Immunosuppressive Drugs" *Encyclopedia of Infection and Immunity* 2022:726–40. doi: 10.1016/B978-0-12-818731-9.00068-9. Epub 2022 Apr 8. PMCID: PMC8987166.

[4] Deguchi K, Zambaiti E, De Coppi P. "Regenerative medicine: current research and perspective in pediatric surgery" *Pediatr Surg Int.* 2023 Apr 4;39(1):167. doi: 10.1007/s00383-023-05438-6. PMID: 37014468; PMCID: PMC10073065.

[5] Wobma H, Vunjak-Novakovic G. "Tissue Engineering and Regenerative Medicine 2015: A Year in Review" *Tissue Eng Part B* Rev. 2016 Apr;22(2):101-13. doi: 10.1089/ten.TEB.2015.0535. Epub 2016 Feb 23. PMID: 26714410; PMCID: PMC4817587.

[6] https://www.biopharma-reporter.com/Article/2019/03/04/Cell-therapy-market-to-triple-in-size-by-2025

[7] Yasaman Esmaeili, Elham Bidram, Ashkan Bigham, Mehdi Atari, Reyhaneh Nasr Azadani, Mohamadreza Tavakoli, Saeideh Salehi, Marjan Mirhaj, Arefeh Basiri, Zahra Mirzavandi, Maryam Boshtam, Mohammad Rafienia, Anousheh Zargar Kharazi, Saeid Karbasi, Laleh Shariati, Ali Zarrabi, "Exploring the evolution of tissue engineering strategies over the past decade: From cell-based strategies to gene-activated matrix" *Alexandria Engineering Journal*

[8] Costela-Ruiz VJ, Melguizo-Rodríguez L, Bellotti C, Illescas-Montes R, Stanco D, Arciola CR, Lucarelli E. "Different Sources of Mesenchymal Stem Cells for Tissue Regeneration: A Guide to Identifying the Most Favorable One in Orthopedics and Dentistry Applications" *Int J Mol* Sci. 2022 Jun 6;23(11):6356. doi: 10.3390/ijms23116356. PMID: 35683035; PMCID: PMC9181542.

[9] McKee C, Chaudhry GR. "Advances and challenges in stem cell culture" *Colloids Surf B Biointerfaces* 2017 Nov 1;159:62-77. doi: 10.1016/j.colsurfb.2017.07.051. Epub 2017 Jul 27. PMID: 28780462.

[10] Rong Z, Wang M, Hu Z, Stradner M, Zhu S, Kong H, Yi H, Goldrath A, Yang YG, Xu

Y, Fu X. "An effective approach to prevent immune rejection of human ESC-derived allografts Cell " *Stem Cell.* 2014 Jan 2;14(1):121-30. doi: 10.1016/j.stem.2013.11.014. PMID: 24388175; PMCID: PMC4023958.

[11] Yang YK. "Aging of mesenchymal stem cells: Implication in regenerative medicine" *Regen Ther.* 2018 Nov 3;9:120-122. doi: 10.1016/j.reth.2018.09.002. PMID: 30525083; PMCID: PMC6222976.

[12] Amer MH, Rose FRAJ, Shakesheff KM, Modo M, White LJ. "Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges" *NPJ Regen Med.* 2017 Aug 10;2:23. doi: 10.1038/s41536-017-0028-x. PMID: 29302358; PMCID: PMC5677964.

[13] Huzum B, Puha B, Necoara RM, Gheorghevici S, Puha G, Filip A, Sirbu PD, Alexa O. "Biocompatibility assessment of biomaterials used in orthopedic devices: An overview (Review)" *Exp Ther Med.* 2021 Nov;22(5):1315. doi: 10.3892/etm.2021.10750. Epub 2021 Sep 17. PMID: 34630669; PMCID: PMC8461597.

[14] Satchanska G, Davidova S, Petrov PD. "Natural and Synthetic Polymers for Biomedical and Environmental Applications" *Polymers (Basel)* 2024 Apr 20;16(8):1159. doi: 10.3390/polym 16081159. PMID: 38675078; PMCID: PMC11055061.

[15] Ghovvati, M., Guo, L., Bolouri, K., Kaneko, N. (2023). Advances in "Electroconductive Polymers for Biomedical Sector: Structure and Properties" *Materials Chemistry Horizons* 2(2), 125-137. doi: 10.22128/mch.2023.681.1038

[16]Yi S, Ding F, Gong L, Gu X. "Extracellular Matrix Scaffolds for Tissue Engineering and Regenerative Medicine" *Curr Stem Cell Res Ther.* 2017;12(3):233-246. doi: 10.2174/1574888X11 666160905092513. PMID: 27593448.

[17] Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R. "Extracellular matrix molecules: potential targets in pharmacotherapy" *Pharmacol* Rev. 2009 Jun;61(2):198-223. doi: 10.1124/pr.109.001289. PMID: 19549927; PMCID: PMC2830117.

[18] Urbanczyk M, Layland SL, Schenke-Layland K. "The role of extracellular matrix in biomechanics and its impact on bioengineering of cells and 3D tissues" *Matrix Biol.* 2020 Jan;85-86:1-14. doi: 10.1016/j.matbio.2019.11.005. Epub 2019 Dec 2. PMID: 31805360.

[19] Gilpin A, Yang Y. "Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications" *Biomed Res Int.* 2017;2017:9831534. doi: 10.1155/2017/9831534. Epub 2017 Apr 30. PMID: 28540307; PMCID: PMC5429943.

[20] Feng Y, Zhu S, Mei D, Li J, Zhang J, Yang S, Guan S. "Application of 3D Printing Technology in Bone Tissue Engineering: A Review" *Curr Drug Deliv.* 2021;18(7):847-861. doi: 10.2174/1567201817999201113100322. PMID: 33191886.

[21] Gopinathan J, Noh I. "Recent trends in bioinks for 3D printing" *Biomater Res.* 2018 Apr 6;22:11. doi: 10.1186/s40824-018-0122-1. PMID: 29636985; PMCID: PMC5889544.

[22] Giannoudis PV, Jones E, Einhorn TA. "Fracture healing and bone repair" *Injury* 2011 Jun;42(6):549-50. doi: 10.1016/j.injury.2011.03.037. Epub 2011 Apr 7. PMID: 21474131.

[23] Weiling Yin, Miao Chen, Jiaxiang Bai, Yaozeng Xu, Miao Wang, Dechun Geng, Guoqing Pan. "Recent advances in orthopedic polyetheretherketone biomaterials: Material fabrication and biofunction establishment" *Smart Materials in Medicine Volume 3*, 2022, Pages 20-36, ISSN 2590-1834.

[24] Charron Frédéric, Tessier-Lavigne, M.. (2009). "Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh)" 10.1016/B978-008045046-9.00334-X.

[25] Kimlin Lauren Kassis, Jareer Virador, Victoria. (2013). "3D in vitro tissue models and their potential for drug screening" *Expert opinion on drug discovery 8.* 10.1517/17460441.2013.852181.

[26] Olowoyeye A, Okwundu CI. "Gene therapy for sickle cell disease" *Cochrane Database Syst Rev* 2018 Nov 16;11(11):CD007652. doi: 10.1002/14651858.CD007652.pub6. Update in: Cochrane Database Syst Rev. 2020 Nov 30;11:CD007652. PMID: 30480767; PMCID: PMC6517046.

[27] Scheller EL, Krebsbach PH. "Gene therapy: design and prospects for craniofacial regeneration" *J Dent Res.* 2009 Jul;88(7):585-96. doi: 10.1177/0022034509337480. PMID: 19641145; PMCID: PMC2907101.

[28] Khalili AA, Ahmad MR. "A Review of Cell Adhesion Studies for Biomedical and Biological Applications" *Int J Mol Sci* 2015 Aug 5;16(8):18149-84. doi: 10.3390/ijms160818149. PMID: 26251901; PMCID: PMC4581240.

[29] Anderson LR, Owens TW, Naylor MJ. "Structural and mechanical functions of integrins" *Biophys* Rev. 2014 Jun;6(2):203-213. doi: 10.1007/s12551-013-0124-0. Epub 2013 Oct 8. PMID: 28510180; PMCID: PMC5418412.

[30] Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. "Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies" *Aging (Albany NY)* 2016 Apr;8(4):603-19. doi: 10.18632/aging.100934. PMID: 27019364; PMCID: PMC4925817.

[31] Shoulders MD, Raines RT. "Collagen structure and stability" *Annu Rev Biochem.* 2009; 78:929-58. doi: 10.1146/annurev.biochem.77.032207.120833. PMID: 19344236; PMCID: PMC 2846778.

[32] Shenoy M, Abdul NS, Qamar Z, Bahri BMA, Al Ghalayini KZK, Kakti A. "Collagen Structure, Synthesis, and Its Applications: A Systematic Review" *Cureus* 2022 May 9;14(5):e24856. doi: 10.7759/cureus.24856. PMID: 35702467; PMCID: PMC9177216.

[33] Youbin Li, Yuzhe Liu, Ronghang Li, Haotian Bai, Zhengqing Zhu, Liwei Zhu, Chenyi Zhu, Zhenjia Che, He Liu, Jincheng Wang, Lanfeng Huang, "Collagen-based biomaterials for bone tissue engineering" *Materials & Design, Volume 210* 2021, doi: 10.1016/j.matdes.2021.110049.

[34] Ricard-Blum S. "The collagen family" *Cold Spring Harb Perspect Biol.* 2011 Jan 1;3(1) :a004978. doi: 10.1101/cshperspect.a004978. PMID: 21421911; PMCID: PMC3003457.

[35] Gallo N, Natali ML, Sannino A, Salvatore L. "An Overview of the Use of Equine Collagen as Emerging Material for Biomedical Applications" *J Funct Biomater.* 2020 Nov 1;11(4):79. doi: 10.3390/jfb11040079. PMID: 33139660; PMCID: PMC7712325.

[36] Zaky AA, Simal-Gandara J, Eun JB, Shim JH, Abd El-Aty AM. "Bioactivities, Applications, Safety, and Health Benefits of Bioactive Peptides From Food and By-Products: A Review" *Front Nutr.* 2022 Jan 20;8:815640. doi: 10.3389/fnut.2021.815640. PMID: 35127796; PMCID: PMC8810531.

[37] Akbarian M, Khani A, Eghbalpour S, Uversky VN. "Bioactive Peptides: Synthesis, Sources, Applications, and Proposed Mechanisms of Action" *Int J Mol Sci.* 2022 Jan 27;23(3):1445. doi: 10.3390/ijms23031445. PMID: 35163367; PMCID: PMC8836030.

[38] Adrián Sánchez, Alfredo Vázquez, "Bioactive peptides: A review" *Food Quality and Safety Volume 1* Issue 1, 1 March 2017, Pages 29–46, https://doi.org/10.1093/fqsafe/fyx006

[39] Rossino G, Marchese E, Galli G, Verde F, Finizio M, Serra M, Linciano P, Collina S. "Peptides as Therapeutic Agents: Challenges and Opportunities in the Green Transition Era" *Molecules* 2023 Oct 19;28(20):7165. doi: 10.3390/molecules28207165. PMID: 37894644; PMCID: PMC10609221.

[40] Halloran D, Durbano HW, Nohe A. "Bone Morphogenetic Protein-2 in Development and Bone Homeostasis" *J Dev Biol.* 2020 Sep 13;8(3):19. doi: 10.3390/jdb8030019. PMID: 32933207; PMCID: PMC7557435.

[41] Shibuya M. Vascular "Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies" *Genes Cancer* 2011 Dec;2(12):1097-105. doi: 10.1177/1947601911423031. PMID: 22866201; PMCID: PMC3411125.

[42] Yang B, Good D, Mosaiab T, Liu W, Ni G, Kaur J, Liu X, Jessop C, Yang L, Fadhil R, Yi Z, Wei MQ. "Significance of LL-37 on Immunomodulation and Disease Outcome" *Biomed Res Int.* 2020 May 16;2020:8349712. doi: 10.1155/2020/8349712. PMID: 32509872; PMCID: PMC7246396.

[43] Yang M, Zhang ZC, Liu Y, Chen YR, Deng RH, Zhang ZN, Yu JK, Yuan FZ. "Function and Mechanism of RGD in Bone and Cartilage Tissue Engineering" *Front Bioeng Biotechnol.* 2021 Dec 15;9:773636. doi: 10.3389/fbioe.2021.773636. PMID: 34976971; PMCID: PMC8714999.

[44] Bellis SL. "Advantages of RGD peptides for directing cell association with biomaterials" *Biomaterials* 2011 Jun;32(18):4205-10. doi: 10.1016/j.biomaterials.2011.02.029. PMID: 21515168; PMCID: PMC3091033.

[45] Kumar VB, Tiwari OS, Finkelstein-Zuta G, Rencus-Lazar S, Gazit E. "Design of Functional RGD Peptide-Based Biomaterials for Tissue Engineering" *Pharmaceutics* 2023 Jan 19;15(2) :345. doi: 10.3390/pharmaceutics15020345. PMID: 36839667; PMCID: PMC9967156.

[46] Hersel U, Dahmen C, Kessler H. "RGD modified polymers: biomaterials for stimulated cell adhesion and beyond" *Biomaterials* 2003 Nov;24(24):4385-415. doi: 10.1016/s0142-9612(03)00343-0. PMID: 12922151.

[47] M Rafiuddin Ahmed, R Jayakumar. "Peripheral nerve regeneration in RGD peptide incorporated collagen tubes" *Brain Research* Volume 993, Issues 1–2, 2003, doi.org/10.1016

[48] Monteiro GA, Fernandes AV, Sundararaghavan HG, Shreiber DI. "Positively and nega-

tively modulating cell adhesion to type I collagen via peptide grafting" *Tissue Eng Part A* 2011 Jul;17(13-14):1663-73. doi: 10.1089/ten.tea.2008.0346. Epub 2009 Jan 27. PMID: 19196133; PMCID: PMC2891269.

[49] Schussler O, Coirault C, Louis-Tisserand M, Al-Chare W, Oliviero P, Menard C, Michelot R, Bochet P, Salomon DR, Chachques JC, Carpentier A, Lecarpentier Y. "Use of arginine-glycine-aspartic acid adhesion peptides coupled with a new collagen scaffold to engineer a myocardium-like tissue graft" *Nat Clin Pract Cardiovasc Med.* 2009 Mar;6(3):240-9. doi: 10.1038/ncpcardio1451. PMID: 19234502.

[50] Dawson J, Schussler O, Al-Madhoun A, Menard C, Ruel M, Skerjanc IS. "Collagen scaffolds with or without the addition of RGD peptides support cardiomyogenesis after aggregation of mouse embryonic stem cells" *In Vitro Cell Dev Biol Anim.* 2011 Oct;47(9):653-64. doi: 10.1007/s11626-011-9453-0. Epub 2011 Sep 23. PMID: 21938587.

[51] Lecarpentier Y, Kindler V, Bochaton-Piallat ML, Sakic A, Claes V, Hébert JL, Vallée A, Schussler O. "Tripeptide Arg-Gly-Asp (RGD) modifies the molecular mechanical properties of the non-muscle myosin IIA in human bone marrow-derived myofibroblasts seeded in a collagen scaffold" *PLoS One* 2019 Oct 1;14(10):e0222683. doi: 10.1371/journal.pone.0222683. PMID: 31574082; PMCID: PMC6772000.

[52] Chimenti I, Rizzitelli G, Gaetani R, Angelini F, Ionta V, Forte E, Frati G, Schussler O, Barbetta A, Messina E, Dentini M, Giacomello A. "Human cardiosphere-seeded gelatin and collagen scaffolds as cardiogenic engineered bioconstructs" *Biomaterials* 2011 Dec;32(35):9271-81. doi: 10.1016/j.biomaterials.2011.08.049. Epub 2011 Sep 3. PMID: 21890193.

[53] Grzesiak JJ, Pierschbacher MD, Amodeo MF, Malaney TI, Glass JR. "Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization" *Biomaterials* 1997 Dec;18(24):1625-32. doi: 10.1016/s0142-9612(97)00103-8. PMID: 9613810.

[54] Zhang L, Hum M, Wang M, Li Y, Chen H, Chu C, Jiang H. "Evaluation of modifying collagen matrix with RGD peptide through periodate oxidation" *J Biomed Mater Res A* 2005 Jun 15;73(4):468-75. doi: 10.1002/jbm.a.30363. PMID: 15900609.

[55] Ren D, Hou S, Wang H, Luo D, Zhang L. "Evaluation of RGD modification on collagen matrix" *Artif Cells Blood Substit Immobil Biotechnol.* 2006;34(3):293-303. doi: 10.1080/10731190600683829. PMID: 16809131.

[F1] Kwan, J.C.; Dondani, J.; Iyer, J.; Muaddi, H.A.; Nguyen, T.T.; Tran, S.D. Biomimicry and 3D-Printing of Mussel Adhesive Proteins for Regeneration of the Periodontium—A Review. Biomimetics 2023, 8, 78. https://doi.org/10.3390/biomimetics8010078

[F2] Gholamali, I.; Vu, T.T.; Jo, S.-H.; Park, S.-H.; Lim, K.T. Exploring the Progress of Hyaluronic Acid Hydrogels: Synthesis, Characteristics, and Wide-Ranging Applications. Materials 2024, 17, 2439. https://doi.org/10.3390/ma17102439

[F3] https://www.stemcures.com/cost-of-stem-cell-therapy

[F4] https://www.pharmasalmanac.com/articles/allogeneic-t-cell-therapies-efficient-commercial-manufacturing-readiness-using-manufacturing-by-design-methodology

[F5] A, Sivashanmugam Rajendran, Arun Kumar Moon, Young Hwang, Nathaniel. (2023). Stimuli-responsive dynamic hydrogel: design, properties and tissue engineering applications. Materials Horizons. 10. 10.1039/D3MH00399J.

[F6] https://skinmanagementsystem.com/blogs/news/how-to-improve-the-collagen-network-in-the-skin-skin-management-system-by-dr-strauss

[F7] Sanchez, Adrian Vázquez, Alfredo. (2017). Bioactive peptides: A review. Food Quality and Safety. 1. 29-46. 10.1093/fqsafe/fyx006.

[F8] Colombo, M.; Bianchi, A. Click Chemistry for the Synthesis of RGD-Containing Integrin Ligands. Molecules 2010, 15, 178-197. https://doi.org/10.3390/molecules15010178

[F9] https://en.wikipedia.org/wiki/1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

[F10] Schussler, O., Coirault, C., Louis-Tisserand, M. et al. Use of arginine–glycine–aspartic acid adhesion peptides coupled with a new collagen scaffold to engineer a myocardium-like tissue graft. Nat Rev Cardiol 6, 240–249 (2009). https://doi.org/10.1038/ncpcardio1451

[F11] Houshmand, Paria Hedayati, Aliakbar. (2022). Dietary Effect of Probiotic and Prebiotic on Some Mucus Indices of Silver Carp (Hypophthalmichthys molitrix) Exposed to Silver Nanoparticles. Biological Trace Element Research. 200. 1-9. 10.1007/s12011-021-03047-9.

[F12] https://link.springer.com/referenceworkentry/10.1007/978-3-030-91346-5$_5$5

[F13] https://mail.almerja.com/more.php?idm=131100

[F14] https://www.quora.com/What-are-the-main-feachers-of-HPLC

[F15] https://www.tissyou.com/portfolio$_page/collygen/$