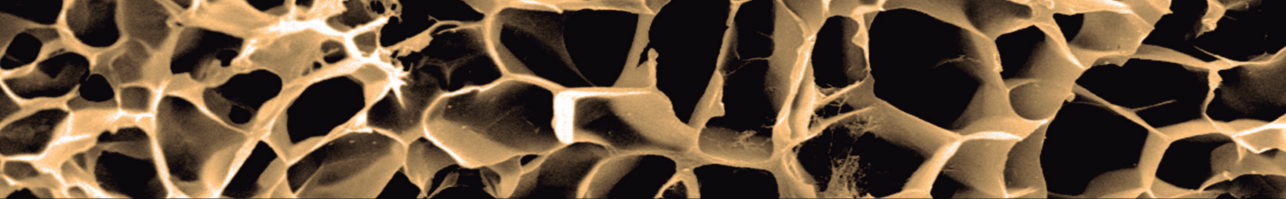

Universidad del País Vasco
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Tailored Gelatin-based three-dimensional systems for bone tissue engineering

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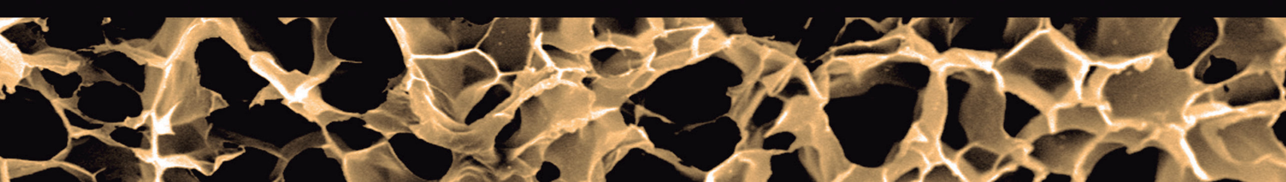

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CAMPUS DE EXCELENCIA INTERNACIONAL

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NanoBioCel Group
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Orain dela lau urte hasitako bidaiari amaiera emateko unea iristear dagoen honetan, bidelagunak izan direnak eskertzeko garaia heldu da. Hitz hauen bitartez, modu batera edo bestera Doktorego Tesi hau aurrera eramaten lagundu didaten pertsona guztiak eskertu nahiko nituzke.

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The last experimental work (Chapter 3) "*Biphasic hydrogels integrating mineralized and anisotropic features for interfacial tissue engineering*" has been sent to the ACS Applied Materials & Interfaces Journal.

I was taught

that the way

of progress

is neither swift

nor easy.

Marie Curie

GLOSSARY / GLOSARIOA

3D: three-dimensional / hiru dimentsiotako

A/A: antibiotic/antimycotic / antibiotiko/antimikotiko

ALP: alkaline phosphatase / fosfatasa alkalinoa

ANOVA: analysis of variances / bariantzen analisia

BCP: biphasic calcium phosphate / kaltzio fosfato bifasikoa

bFGF: basic fibroblast growth factor / fibroblastoen hazkuntza faktore basikoa

BMP-2: bone morphogenetic protein-2 / hezur-proteina morfogenikoa-2

BMP-4: bone morphogenetic protein-4 / hezur-proteina morfogenikoa-4

BM-MSCs: bone marrow derived mesenchymal stem cells / hezur-muineko zelula-ama mesenkimalak

CCK-8: Cell Counting Kit-8 / zelulak kontatzeko kit 8

CNC: cellulose nanocrystals / zelulosazko nanokristalak

CNT: carbon nanotubes / karbonozko nanohodiak

DAPI: 4,6-diamidino-2-phenyindole dilactate / 4,6-diamidino-2-fenindol dilaktatoa

DMEM: Dulbeccos's modified Eagle's medium / Dulbeccok eraldatutako Eagleren medioa

EE: encapsulation efficiency / kapsularatze eraginkortasuna

ECM: extracellular matrix / matrize extrazelularra

EMEM: Eagle's Minimum Essential Medium / Eagleren gutxieneko funtsezko medioa

FBS: foetal bovine serum / behi fetuen seruma

FDA: Food and Drug Administration / farmako eta elikagaien administrazioa

FWHM: full width at half medium / altuera ertaineko zabalera

GO: graphene oxide / grafeno oxidoa

GRAS: generally recognized as safe / orokorrean seguru bezala onartua

HA: hydroxyapatite / hidroxiapatita

hASCs: human adipose-derived stem cells / giza gantz ehunetik eratorritako zelula amak

hMSCs: human mesenquimal stem cells / giza zelula-ama mesenkimalak

HUVEC: human umbilical vascular endothelial cells / giza-zilbor zaineko zelula endotelialak

IGF-1: insulin-like growth factor-1 / insulinaren-antzeko hazkuntza faktorea-1

μ-CT: micro-computed tomography / tomografia mikro-konputatua

MSCs: mesenchymal stem cells / zelula-ama mesenkimalak

MMP: matrix metalloproteinases / matrizearen proteinasa metalikoak

mTG: microbial transglutaminase / transglutaminasa mikrobiarra

OCP: octacalcium phosphate / oktakaltzio fosfatoa
OPF: oligopolyethylene glycol fumarate / oligopolietilenglikol fumaratoa
OPN: osteopontin / osteopontina
PANI: polyaniline / polianilina
PBS: phosphate buffered saline / gatz fosfato tanpoia
PCL: polycaprolactone / polikaprolaktona
PLA: polylactic acid / azido polilaktikoa
PLGA: poly(lactic-co-glycolic) acid / azido poli(laktiko-ko-glikolikoa)
pNPP: p-nitrophenyl phosphate / p-nitrofenil fosfatoa
PPF: polypropylene fumarate / polipropilen fumaratoa
R^2: regression coefficient / Erregresio koefizientea
RGD: arginine-glycine-aspartic acid / arginine-glizina-azido aspartikoa
SD: standard deviation / desbideraketa estandarra
SEM: scanning electron microscopy / ekorketazko mikroskopio elektronikoa
SMCs: smooth muscle cells / muskulu leuneko zelulak
TCP: tricalcium phosphate / trikaltzio fosfatoa
TNC: tenascin / tenaszina
VEGF: vascular endothelial growth factor / endotelio baskularraren hazkuntza faktorea

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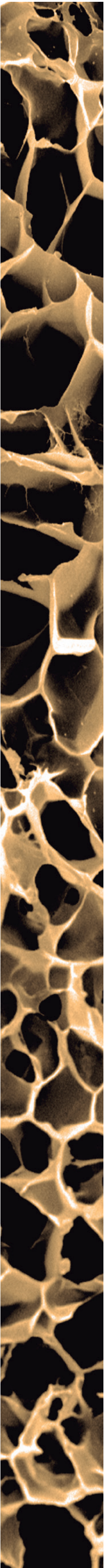

Introduction

Gelatin as Biomaterial for Tissue Engineering

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ABSTRACT

Tissue engineering is considered one of the most important therapeutic strategies of regenerative medicine. The main objective of these new technologies is the development of substitutes made with biomaterials that are able to heal, repair or regenerate injured or diseased tissues and organs. These constructs seek to unlock the limited ability of human tissues and organs to regenerate. In this review, we highlight the convenient intrinsic properties of gelatin for the design and development of advanced systems for tissue engineering. Gelatin is a natural origin protein derived from collagen hydrolysis. We outline herein a state of the art of gelatin-based composites in order to overcome limitations of this polymeric material and modulate the properties of the formulations. Control release of bioactive molecules, formulations with conductive properties or systems with improved mechanical properties can be obtained using gelatin composites. Many studies have found that the use of calcium phosphate ceramics and diverse synthetic polymers in combination with gelatin improve the mechanical properties of the structures. On the other hand, polyaniline and carbon-based nanosubstrates are interesting molecules to provide gelatin-based systems with conductive properties, especially for cardiac and nerve tissue engineering. Finally, this review provides an overview of the different types of gelatin-based structures including nanoparticles, microparticles, 3D scaffolds, electrospun nanofibers and *in situ* gelling formulations. Thanks to the significant progress that it has already been made, along with others that will be achieved in a near future, the safe and effective clinical implementation of gelatin-based products is expected to accelerate and expand shortly.

Keywords: biomaterial, gelatin, 3D scaffolds, tissue engineering, regenerative medicine, particles, bone.

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1. INTRODUCTION

There is an increasing clinical need for fabrication of new technologies and biomaterial-based approaches to heal, repair or regenerate injured or diseased tissues and organs. This demand is enormous for all types of tissues but especially for those related to chronic and acute musculoskeletal conditions, including bone, cartilage, tendon, ligament trauma and other injuries, degenerative disc disease and osteoarthritis among others. The treatment of these and other conditions involves 34 million surgical procedures per year only in the USA [1].

The current shortage in tissue and organ donor supply together with the often severe immune complications related to organ transplantation have fueled new scientific disciplines such as tissue engineering [2] and regenerative medicine. The latter is an interdisciplinary field that applies biological and engineering principles to the design and development of technologies that promote regeneration to restore diseased and injured tissues and whole organs [3]. There are a number of strategies to address this issue. Of particular relevance is the fabrication of biomaterial-based scaffolds that are used to substitute temporary the natural tissue while promoting its regeneration. Scaffolds are considered temporary artificial extracellular matrices, one of the three components comprising the tissue engineering triad, along with the cells and the biological factors. These implantable porous structures act as cell delivery, drug delivery or dual delivery carriers. Furthermore, an ideal scaffold should be designed to have several characteristics. For example, these constructs must be biocompatible causing minimal immunological and inflammatory responses, biodegradable in a controllable rate that approaches the tissue regeneration process and highly porous. In the case of cell delivery scaffolds, the pores are necessary to allow cell attachment, penetration, proliferation and extracellular matrix (ECM) deposition. The function of the scaffolds is to guide the growth of cells that have been seeded on the structure or cells that have migrated from surroundings tissues. Besides, the interconnectivity between pores is essential to achieve high cell seeding density and to make easier the exchange of nutrients and waste products. Regarding the mechanical properties, these should be similar to native tissue and provide protection to cells from tensile forces. Surface chemistry and topography features play a key role in fostering cellular interactions and tissue development [4].

It is widely known that biomaterials and biomaterial-based scaffolds play key roles in modern regenerative medicine approaches as optimized milieus that drive cellular fate and function [5]. The recent years have marked a substantial paradigm shift in the design and fabrication criteria for modern biomaterials, fully integrating principles from biology, biomedical engineering and pharmaceutical technology.

Gelatin is a natural biopolymer that is widely used in pharmaceutical, cosmetic and medical fields because of its unique biological and technological properties. In the pharmaceutical and medical applications, gelatin has been used as a matrix for implants, in injectable drug delivery systems, as a stabilizer in diverse vaccines, in the manufacturing of soft and hard capsules, as plasma expanders, sealants, wound care and hemostats [6-8]. Nevertheless, in the last years, a great advance has been made in the efforts carried out in order to integrate gelatin as a useful biomaterial in the design of structures for tissue engineering.

In this review, we highlight herein the central role of gelatin as a biomaterial in tissue engineering for tissue repair and regeneration. This review provides a state-of-the art overview of gelatin and gelatin composites for their use in regenerative medicine strategies. We discussed the potential clinical application of gelatin-based formulations including micro and nanoparticles, scaffolds and *in situ* gelling products. For a more comprehensive understanding of the field as well as its successful application in biomedicine we refer to several outstanding recent reviews.

2. PROPERTIES OF GELATIN

Gelatin is a natural origin protein derived from chemical, physical or enzymatic hydrolysis of collagen type I, the main protein component of the skin, bones and connective tissue of animals, including fish and poultry. Collagen is composed of interconnected protein chains. In the hydrolytic process, the collagen characteristic triple helix structure is broken and single-stranded macromolecules are obtained.

2.1 Structure of gelatin

The primary structure of gelatin is formed by over twenty different amino acids in variable proportions (Figure 1). The amino acid composition and the corresponding sequence vary depending on its origin, and this, in turn, influences the final properties of gelatin. From the structural point of view, gelatin is composed of Glycine-X-Y peptide triplets repetitions, where, theoretically, X and Y can be any amino acid, but proline for X and hydroxyproline for Y positions are the most common. Gelatin might be considered as a mixture of amino acid moieties joined by peptide bonds forming polymers ranging in molecular weight between 15,000 and 400,000 Daltons. Importantly, the length of the polypeptide chains depends on the processing parameters (temperature, time and pH), the pretreatment method and the properties of the raw material [9]. In fact, during the hydrolytic process, the three polypeptide chains forming the triple helix of collagen are broken and one polymer chain (α-chains), two α-chains covalently crosslinked (β-chains) and three covalently crosslinked α-chains (γ-chains) are obtained. The proportion of each type of chains obtained is different depending on the above mentioned variables, and this makes for differences in molecular weight of amino acid chains. There are strong non-covalent interactions between gelatin

molecules, such as hydrogen bonds, van der Waal forces and electrostatic and hydrophobic interactions [10]. Therefore, there are several types of gelatin with different composition depending on the source of collagen used and the hydrolytic treatment employed. The entire process for obtaining gelatin has three general phases: Pretreatment of the raw material and collagen isolation, extraction of the gelatin and purification and drying [11].

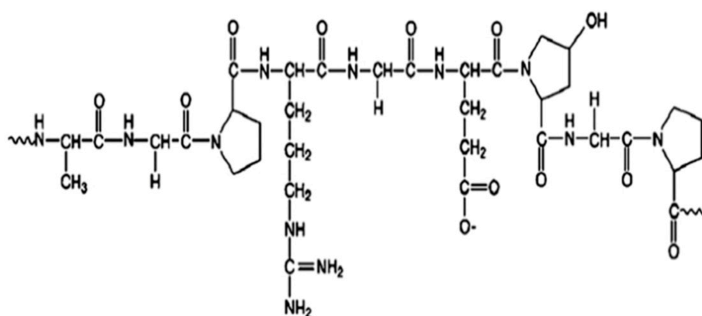

Figure 1. Chemical structure of gelatin. Reproduced, with permission, from [141].

2.2 Gelatin sources

Regarding the source, there are also different types of gelatin. Mammalian derived gelatins have been the most popular and widely used for regenerative purposes and, predominantly, these gelatins are obtained from pig and bovine, especially from skin and bones [12]. Nevertheless, these materials are not the ideal prototype of biomaterial in the field of tissue engineering, especially because of socio-cultural and health related concerns. The risk of disease vectors transmission must be taken into account, such as prions and bovine spongiform encephalopathy. Although there are studies carried out by different authorities in order to demonstrate that the process of obtaining gelatin from mammalian collagen is an effective method to eliminate the presence of possible prions in the raw material [11]. In the past decade other sources of gelatin, including fish, poultry and vertebrates have been introduced with the main objective of providing alternatives to mammalian derived gelatins. However, due to law concerns, poultry derived gelatins commercial production is still limited nowadays. Therefore, fish derived gelatin may be a better alternative to mammalian gelatins. Gelatins from warm and even cold water fish skins, bones and fins have been produced since 1960 using a number of different methods [13-17]. Even though gelatins derived from fish have related features to porcine gelatins, the amino acid sequence and proportions are different. In fact, fish derived gelatin has lower content of amino acids (proline and hydroxyproline) that are responsible for fixing the ordered structure when forming a gel network. This makes differences in some properties such as the melting temperature, gelling temperature, gel modulus, thermal stability and viscosity. Fish derived gelatin has a significantly lower

melting temperature, lower thermal stability and higher viscosity. One important challenge for gelatin material is the potential development of allergic reactions [18].

In order to overcome the disadvantages and improve properties of tissue-derived materials, recombinant gelatins have been developed. With this technology, it is possible to produce gelatins with defined molecular weight and isoelectric point, with the advantage of increasing the reproducibility between batches. For the production of human recombinant gelatin, two different techniques have been developed. In one of the approaches, there is the possibility of expressing recombinant collagen, purified and denatured (with or without chain fragmentation) to obtain recombinant gelatin as a final product. On the other hand, specific α-chains can be produced directly. There have been various expression systems used for the production of recombinant gelatin such as *Pichia pastoris* or *Hansenula polymorpha* yeast, *Escherichia coli* or even transgenic systems like tobacco plant or mice [19].

2.3 Biological properties

In relation to the properties of gelatin, some of them are common to all gelatins, while many others depend on the source and the method used to obtain them. In this regard, it has been found that gelatin is a biocompatible material, not cytotoxic and with low immunogenicity compared to collagen. This material is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) [20]. Gelatin contains in its structure biological functional moieties of arginine-glycine-aspartic acid (RGD) sequences that improve the adhesion, differentiation and proliferation of cells [21]. In addition, it is a biodegradable material because it has sensitive sites for enzymatic degradation by matrix metalloproteinases (MMP). Finally, the products obtained through the enzymatic degradation are themselves biocompatible [22]. As for the physicochemical properties of the gelatin, it is a water soluble polypeptide having an amphoteric behavior, because of the presence of alkaline amino acids and acid functional groups [23]. However, its electrostatic charge depends on the treatment used for its extraction, which gives gelatins with different isoelectric points, as mentioned above.

2.4 Physicochemical properties

2.4.1 Isoelectric point

Depending on the method used for collagen pretreatment prior to the extraction process, two types of gelatin are predominantly produced: Type A and Type B. Gelatin Type A, with an isoelectric point of 9.0, is derived from collagen acidic hydrolysis, using sulfuric or even hydrochloric acid. Gelatin derived from an alkaline treatment, with an isoelectric point of 5.0, is known as Type B. In this case, asparagine and glutamine amide groups are hydrolyzed into carboxyl groups resulting on aspartate and glutamate residues [24].

2.4.2 Thermoreversible property

One of the most significant properties of gelatin is the ability to form thermoreversible gels in water, due to the conformational transition that presents after cooling the solution. Gelatin granules form swollen particles when they are hydrated in cold water, and then by heating, these swollen particles dissolve forming a solution. During the gelation process, locally ordered regions are formed, which are subsequently joined by non-specific bonds [25]. These intermolecular interactions are usually hydrophobic, hydrogen and electrostatic bonds. Thermoreversible polymer gel can be defined as gel formed due to the entanglement of polymer chains whose viscosity changes at a characteristic temperature of gelation [26]. Because of the bonding energy in gelatin gels is relatively weak, the physical gels are thermally reversible [25]. The behavior of the gelatin solution depends on different factors: temperature, pH, method of production, thermal history and concentration. Gel strength and viscosity of gelatin are reduced by heating the solution above 40 °C. However, the rigidity of the gel does not depend only on temperature, since its concentration, intrinsic strength (bloom strength), pH and the presence of any additive can also modify it [27]. The bloom strength is the measurement of the strength of the physical gel that is formed upon cooling [28]. Gelatin structure together with the molecular mass defines this value.

2.4.3 Crosslinking of gelatin

The lack of thermal stability at physiological temperature is the main drawback of gelatin for its use in medical applications, such as in tissue engineering. Therefore, a crosslinking agent is needed when using gelatin as a biomaterial. Gelatin crosslinking improves thermal and mechanical stability of this material under physiological conditions and decrease the ratio of *in vivo* degradation [29]. Depending on the type of crosslinking agent used to reticulate gelatin, physical, mechanical and cytotoxic properties of the hydrogel will be different. The methods employed for crosslinking gelatin can be classified into two general groups: physical and chemical crosslinking. In the physical crosslinking technique there is no need for making any modification or using any chemical agent that can be toxic, but it is difficult to control the crosslinking density and, often, the procedure is less efficient [30]. Microwave energy [31], dehydrothermal treatment [32-34] and ultraviolet radiation [35] have been used to achieve physical crosslinking. By contrast, in the chemical crosslinking strategy, chemical agents are used in order to crosslink gelatin strands. Some agents, that can be bifunctional or polyfunctional, are incorporated between gelatin molecules forming chemical bonds with the free amino groups of lysine and hydroxylysine or carboxyl groups of glutamate and aspartate [30]. During the degradation of the crosslinked gelatin, reactive and toxic reagents can be released into the body and may cause damage [36]. Examples of crosslinking agents that follow this process are aldehydes (glutaraldehyde [37] or glyceraldehydes [38]), polyepoxides and isocyanates [39]. These agents are known as non-zero lengths crosslinkers. However, other agents

activate the carboxyl groups of the gelatin molecules to react with the amino groups of adjacent protein chains forming intramolecular amide bonds. In this case, the molecules of the agents, known as zero-length crosslinkers, are not introduced into the final structure [30].

Traditionally, aldehydes have been the agents most frequently used for crosslinking gelatin. However, they are not suitable for encapsulating cells due to the cytotoxicity, immunogenicity and inflammatory effects associated with products obtained by its degradation. Therefore, increasing interest has been devoted to natural agents with less cytotoxic effects to crosslink gelatin hydrogels. For example, genipin is a natural crosslinker isolated from the fruits of *Gardenia jasmoides* plant that has been widely used to crosslink several materials for biomedical applications. Interestingly, it has low cytotoxicity, high crosslinking efficiency and good biocompatibility [40,41].

On the other hand, enzymatically crosslinked hydrogels are emerging for tissue engineering, since these reactions are carried out under physiological conditions and substrate specificity is achieved. Most enzymatic reactions are catalyzed at neutral pH, in an aqueous medium and at moderate temperature [42]. Hydrogels have been prepared by using enzyme systems such as tyrosinases, transferases and peroxidases [43]. In the case of gelatin, crosslinking using microbial transglutaminase [44,45] and mushroom tyrosinase [46,47] have been effectively applied for the development of formulations.

To date, the most common approach to crosslink gelatin has been done without any prior modification. Nevertheless, there is the possibility of including previous modifications on the gelatin molecules to improve the control over the crosslinking process. In this case, functional groups are added to side groups of gelatin and a better degree of control over the design and properties of the final hydrogel is achieved. Different functional groups have been used for gelatin modification such as acrylamide, norborene, methacryloyl or even ferulic acid [28]. In this regard, gelatin methacryloyl seems to be the most investigated approach for various biomedical applications as this modification does not affect the RGD and MMP-sensitive motifs [36,48].

3. GELATIN-BASED COMPOSITES

As already described above, gelatin shows many suitable properties that make it an interesting biomaterial to be used in tissue engineering for different purposes. However, recently, great efforts have been made to develop gelatin-based composite systems, in order to fill some gaps that this biomaterial might have. The aim has been to seek synergies between materials with different properties, in order to achieve composite formulations with improved properties that address the particular tissue engineering requirements. Therefore, over the past few years more advanced formulations with two or more materials have been

developed [20]. In this regard, the objectives to be achieved with the development of composite formulations have been, among others, the improvement of the mechanical properties of gelatin formulations, the development of new formulations with conductive properties and the ability to control the release of bioactive molecules (Table 1).

3.1 Improvement of the mechanical properties

Due to the high water content of hydrogels, their mechanical strength tends to be limited and this might compromise their use in tissues presenting challenging mechanical conditions, such as bone [49] and cartilage [50,51]. In the case of gelatin [52], although these properties improve with the use of crosslinking agents, the necessary requirements are not often achieved [53]. In recent years, different approaches have been carried out to overcome these limitations. One of them has been to integrate a second material in the scaffold design in order to fabricate a hybrid material composite [54].

The bone itself can be considered a natural composite material which is formed by an organic phase and a mineral one. The organic phase consists mainly on type I collagen and the mineral phase is, essentially, calcium phosphate, especially hydroxyapatite (HA) [55]. Calcium phosphate is responsible for the bone compressive strength and collagen gives resilient properties, making the tissue tough and elastic [56]. Hence, combining compounds that improve the mechanical properties that the gelatin lacks is especially interesting for the development of formulations targeted to bone regeneration, because bone tissue mimetic constructs are achieved. The mechanical properties (elastic modulus, tensile strength, fracture toughness, fatigue and elongation percentage) of the scaffold depend on the materials used to fabricate it and these properties should be similar to those of the bone that we want to regenerate or replace. In fact, bone loss, osteopenia and stress shielding are injuries associated with the use of bone grafts. With the similarity of the properties, the mechanical compatibility will be achieved, but taking into account that bone mechanical properties might differ from cortical bone to cancellous bone, it is not easy to design an ideal bone scaffold [57]. Recently, diverse composite systems have been designed and fabricated combining gelatin with ceramics, natural origin polymers or synthetic polymers, as well as mixtures of these materials [58].

3.1.1 Calcium phosphate ceramics

Calcium phosphate ceramics are bioactive materials composed of calcium ions and orthophosphates, metaphosphates or pyrophosphates. These compounds are of great interest in bone tissue engineering, not only because they increase the required mechanical properties of formulations providing strength to polymeric scaffolds, but also because most of them have osteoconductive properties (supporting osteoblasts adhesion and proliferation) and some of them have also shown osteoinductive properties (stimulating new bone formation by recruiting progenitors or inducing differentiation towards osteoblastic lineage). Solubility

properties, crystallinity and calcium to phosphate ratio make differences between these ceramic compounds in relation to the capacity of promoting ossification [59-61]. Calcium phosphate ceramics have been used in combination with gelatin to create a composite system that meets all the necessary properties to get an appropriate construct to be used in bone tissue engineering (Figure 2). Regarding the most important features that a substitute for bone tissue engineering should ideally have, osteoinduction and osteoconduction ability and similar modulus of elasticity, tensile strength, fatigue, and the percentage of elongation of the bone to be replaced or regenerated seem to be the desirable properties [62]. HA [63-69], tricalcium phosphate (TCP) [70-74], biphasic calcium phosphate (BCP) [75-78] and octacalcium phosphate (OCP) [79-81] are some of them. Also, there are several formulations where calcium phosphates are combined in order to make better scaffolds [82].

HA is one of the most commonly used calcium phosphate ceramics for bone tissue engineering, a crystalline calcium phosphate ($Ca_{10}(PO^4)_6(OH)_2$) derivative present in the inorganic component of bone matrix. Besides, one of the most important composites studied for bone tissue engineering is a blend of HA with gelatin [83]. In fact, there are lots of gelatin-based formulations combined with HA. Some systems are composites only of two phases, but there are some more advanced systems that include various types of polymers with HA [63].

As an example, in order to develop modularly engineering biomimetic osteon, HA and gelatin have been combined in different proportions. A hydrogel with higher mechanical rigidity and better biocompatibility has been successfully fabricated. Then, a structure with two different rings mimicking osteons was manufactured. In the inner ring, Human Umbilical Vascular Endothelial Cells (HUVECs) were grown to mimic blood vessels while the external rings encapsulated osteoblast-like cells (MG63) [65].

On the other hand, BCP are ceramics with two phases that present a low solubility apatite phase and a more soluble phase with osteoinductive properties [84]. One method for combining these materials is the use of nanoparticles. In order to get nanofibrous scaffolds with supportive mechanical strength for ideal bone regeneration, the inclusion of BCP nanoparticles helped to get further stability. Importantly, this new combined formulation showed improved not only mechanical but also enhanced biological properties, measured as osteoblast adhesion and osteopontin osteogenic protein expression, compared to scaffolds without ceramics. This improvement on the characteristics of the scaffold was evaluated not only with *in vitro* studies, but also with *in vivo* studies using a rat calvaria model, where it was observed an increase in bone formation on week 2 and 4 [75].

Finally, material biodegradation is an aspect that must be taken into account when using calcium phosphate ceramics. In fact, different resorption mechanisms have been proposed for ceramics used in bone regeneration [57]. Chiba S. *et al.* studied the resorption rate and

osteoconductivity of calcium phosphate materials. Biodegradation rate and quality of regenerated bone were compared among four different materials. Complete resorption was observed in the OCP-Gelatin composite, while commercially available TCP implants were not completely resorbed [79].

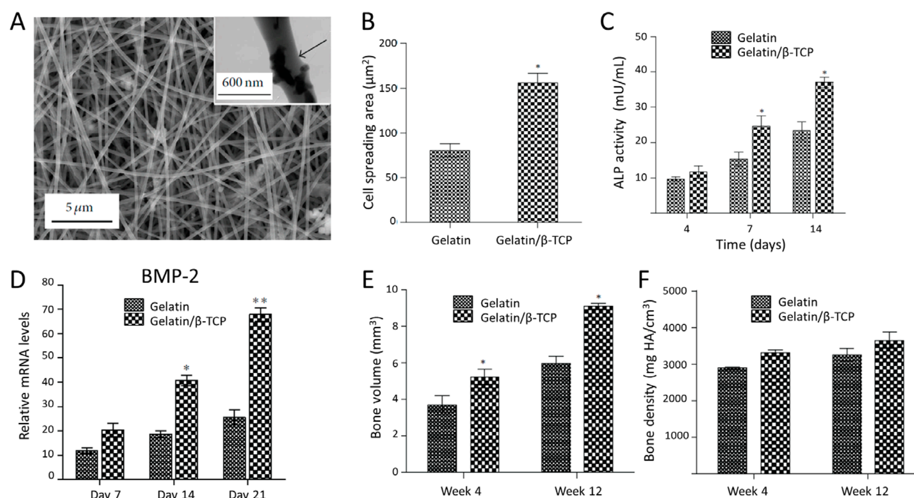

Figure 2. **Electrospun gelatin/β-TCP composite nanofibers for osteogenic differentiation of bone marrow mesenchymal stem cells (BM-MSCs) and for enhance *in vivo* bone formation**. (A) SEM and TEM image of electrospun gelatin/β-TCP composite nanofibers before crosslinking. (B) Cell spreading areas and (C) ALP activity of BM-MSCs cultured on various scaffolds at 4, 7, and 14 days. (D) The mRNA expression level of BMP-2 osteogenic gene in BM-MSCs cultured on electrospun nanofibrous scaffolds. The quantitative analysis of the bone volume (E) and bone density (F) at 4 weeks and 12 weeks after scaffolds were implanted on rat calvarial defects. (*$p<0.05$) Adapted from [71].

3.1.2 Synthetic polymers

Synthetic origin polymers have also been used over the last years in tissue engineering for different purposes. It is a large group of materials with very different mechanical properties, but all of them can be synthesized under controlled conditions and their mechanical and physical properties are predictable and reproducible. However, their bioactivity is limited because their surface is usually hydrophobic [85]. Therefore, combination of synthetic polymers with properties that will complement gelatin matrices is of great interest for designing systems that meet the requirements of the tissue that we want to replace. The synthetic polymers would provide structural functionalities and the gelatin, as a natural polymer, supplies biological properties to the scaffold [86].

Polylactic acid (PLA) [86-90] and polycaprolactone (PCL) [35,91-93] are two of the most widely used synthetic polymers to fabricate scaffolds in order to improve the mechanical properties of gelatin-based matrices. Lactic acid is a chiral molecule that has two optically active forms: L-lactide and D-lactide. Through the polymerization of these molecules, a

semicrystalline polymer is formed. The mechanical properties of the polymer are influenced by the molar mass and the degree of crystallinity. PLA is a biodegradable polymer with slow degrading time that has been widely studied for several medical applications such as drug delivery systems, absorbable sutures and implants [94]. On the other hand, PCL is a linear and hydrophobic synthetic polymer with high mechanical strength and poor hydrophilicity [92]. Both are biodegradable and biocompatible, they show good mechanical properties and present the advantage of easy access and processability [95].

In recent years, electrospinning natural and synthetic polymer blends, such as gelatin with PLA or PCL, have been extensively used to fabricate composite nanofibers [96]. Additionally, as it was concluded in a work carried out with gelatin-PCL hybrid scaffolds, composite nanofibers must be morphologically uniform and compositionally homogeneous [97]. Another aspect to be considered when fabricating hybrid nanofibers is the blend ratio used, since both mechanical and biological performances of the nanofibrous scaffolds will depend on this parameter. A correlation between the best mechanical properties and a better mesenchymal stem cells (MSCs) behavior has been described. This effect might be due to the fact that the mechanical property is an integral part of the physical microenvironment of cells and, therefore, it affects cellular functions [91].

Recently, tubular composite scaffolds with aligned fibers ranged from 100 to 500 nm of poly (L-lactic acid)/gelatin were designed for vascular tissue engineering and fabricated using the electrospinning technique. Different assays demonstrated the enhancement of viability and proliferation of HUVECs and Smooth Muscle Cells (SMCs) proportionally to the gelatin content. These results suggest that cellular organization improves with the alignment of the fibers inside the scaffolds. Importantly, this formulation can be useful to get blood vessels with essential mechanical properties and organization [89].

However, in order to achieve synergies between properties of hybrid composite materials, interactions between substrates are necessary. The introduction of chemical bonds is sufficient to improve the composite strength. In a recent study, photopolymerization technique was used to bind covalently PCL and gelatin with the objective of enhancing the mechanic properties of cartilage implants. With this purpose, PCL was functionalized with methacrylated groups and covalently linked to gelatin methacrylamide [54].

3.2 Composites with conductive properties

Scaffolds with conductive properties have been extensively used in various biomedical fields such as nerve [98], cardiac [99] and bone [100] tissue engineering. In these tissues, electrical signals perform critical function as physiological stimuli, controlling the adhesion and differentiation of cells. For that reason, the use of conductive materials to produce scaffolds seems to be a promising strategy [101]. In fact, these materials could provide

electrical impulses to cells and promote improvements in cell functions, including adhesion, proliferation, migration and differentiation [102]. Actually, electrically conductive scaffolds have been designed as a skeleton of stem cell niche to assist electro-active tissue recovery and regeneration, serving as a pattern to guide and modulate stem cell-specific differentiation, even without applying induction factors [103].

Traditionally, conductive materials have been generally classified into three main categories: conductive polymers, carbon-based materials and metals. In recent years, conductive composites have been designed to overcome the limitation of single conductive material and thus, improve the general performance of materials via the synergistic effect. Many of these materials have been used in combination with natural polymers, such as gelatin, to obtain scaffolds with excellent conductivity [104].

3.2.1 Polyaniline

Polyaniline (PANI), the oxidative polymeric product of aniline under acidic conditions, is one of the most studied conductive polymers in regenerative medicine. It can be polymerized from aniline chemically and electrochemically. This conductive polymer is easy to synthesize and it has been demonstrated that it is compatible with specific cell types [98]. Nevertheless, in order to enhance its biocompatibility and cellular adhesion, it has been combined with different materials, such as with gelatin, in order to form conductive hydrogels [105-108].

As an example, in a study of gelatin/PANI nanofibers in order to investigate its potential as a fibrous conductive scaffold for tissue engineering, it was seen that the proliferation and morphology of cardiac myoblasts H9c2 were similar to those seeded onto control glass coverslips and tissue cultured-treated plastic surfaces [109]. These results opened the way to further demonstrate its use as a fibrous matrix to support cell growth.

Recently, in another research work carried out with gelatin and PANI in combination, the proliferation of Schwann cells and *in vitro* biodegradation behavior was analyzed. The designed and developed formulation showed appropriate conductivity, mechanical properties and biocompatibility. In this novel porous conductive scaffold, the matrix was based on a combination of gelatin and chitosan, while nanoparticles with PANI and graphene were incorporated to give conductive properties. The results concluded that it might be an interesting candidate for potential application in peripheral nerve repair [110].

Promisingly, Yibo *et al.* have designed a novel bioactive scaffold improving its electrical properties while maintaining the physical and biocompatible properties (swelling, compression modules, cell adhesion and spreading responses) of pure gelatin methacrylate scaffolds. The strategy followed to develop a conductive hybrid composite was to use interfacial polymerization of aniline monomers within gelatin methacrylate. In addition, in this interesting research work, the developed composite hydrogel was printed in defined complex geometries using

digital stereolithography. This approach can be used with different photosensitive hydrogels, thus, improving the development of new bioelectrical interfaces [111].

3.2.2 Carbon-based nanosubstrates

Carbon-based compounds are conductive materials that have been investigated for their use in tissue engineering. Carbon nanotubes (CNT) and graphene oxide (GO) are two of them [103].

These materials have unique physical, chemical and mechanical properties that make them interesting compounds for use as platforms for stem cells therapies and tissue engineering [112]. The combination of these substrates with biomaterials that improve the biological properties of the systems, such as gelatin, is an interesting strategy to optimize formulations for tissue engineering, especially for cardiac and nerve tissue engineering [113,114].

3.2.2.1 Graphene oxide (GO)

Graphene is a macromolecular nanomaterial with high robustness and flexibility, composed of carbon atoms in a single two-dimensional layer. This material has extraordinary physicochemical properties, exceptional mechanical, electrical and optical properties and it also has demonstrated biocompatibility [115]. GO is one of the chemical derivatives of graphene that has been obtained by oxidation and exfoliation of graphite [116]. It has lots of hydrophilic functional groups and, because of this, it disperses in aqueous media. Also, it is possible to modify it chemically which makes it useful for biomedical applications [117].

Over the last years, various types of hybrid formulations have been developed combining gelatin and GO in order to obtain systems with conductive and regenerative properties [118-123]. This combination of materials has been used as an hybrid system for non-viral gene therapy in the myocardium. In order to promote vasculogenesis and cardiac repair, an injectable hydrogel based on the combination of methacrylated gelatin and GO nanocomplexes has been investigated. This nanocomplex is a polyethyleneimine delivery system functionalized with GO nanosheets that complexed with the gene encoding the pro-angiogenic factor Vascular Endothelial Growth Factor (VEGF). Using this formulation, the application of gene therapy in a controlled and localized way has been achieved. *In vivo* studies concluded a significant increase in myocardial capillary density in the infarct region where the formulations were injected. In addition, this observation was accompanied by a better performance in echocardiography [121]. Taking into account that in this *in vivo* study the therapeutic efficacy and biocompatibility of the formulations showed to be suitable, the development of these composite systems can be considered as a promising advance in acute myocardial infarction therapy. Besides, in several studies carried out with formulations combining gelatin and GO, it has been found that the incorporation of GO not only adds electrical conductive properties necessary for the regulation of heart cells behavior but also, it helps to improve the mechanical

properties and creates useful additional pores for a better diffusion of nutrients and waste products through the matrix [120].

This research work has not been the only one that has concluded that the addition of GO to the gelatin matrix increases its mechanical strength. For instance, in another study, it has been verified how the incorporation of GO affects the mechanical strength and the osteogenic differentiation. The results confirmed a significant increase in the mechanical properties (compressive and yield strength) of gelatin/HA matrices and osteogenic differentiation of human adipose MSCs induced by the incorporation of GO. The level of differentiation was the same that the one achieved with the use of supplements in the media [123].

Therefore, a combination of gelatin and GO is of great interest as it was concluded in the study conducted by Shin *et al*. In this paper, hybrid hydrogel consisting of methacrylated gelatin and reduced GO was developed in order to achieve myocardial tissue constructs. Cardiomyocytes were seeded in that composite system and results showed improvement on cell viability, proliferation and maturation, and stronger contractility comparing with those of pure gelatin matrices [122].

With these results, it can be concluded that the addition of GO to gelatin-based matrices adds, not only conductivity but also improves the mechanical properties of the scaffold and the osteogenic differentiation of MSCs.

3.2.2.2 Carbon nanotubes (CNT)

CNT are another carbon-based substrates that have been incorporated into natural origin polymers in order to achieve formulations with reinforced structure and novel properties, including electrical conductivity. It seems that the cytotoxicity associated with CNT, depends on the way it is employed, being toxic as a suspension in culture media, whereas immobilization into matrices seems to be non-toxic. To reduce these adverse effects, the chemical functionalization of their surface can be a useful strategy [124]. It has been described that CNT improve cell adhesion, change cellular morphogenesis and signaling pathways, reduce materials degradation rate and modulate mechanical properties [125].

In a recent research work, it has been found that the development of hybrid gelatin nanofibers scaffolds with multi-walled CNT can be useful in the formation of myotubes, in order to meet the functional requirements demanded in muscle tissue engineering. When the main objective is to design a functional skeletal muscle tissue construct, it is very important that myoblasts seeded on scaffolds mimicking the ECM are aligned. An interesting strategy to align cells and improve their contractility is to use aligned nanofibers, such as in the formulation used by Ostrovidov *et. al.*, where gelatin and aligned multi-walled CNT electrospun hybrid scaffolds were designed [126]. With this system, it was possible to enhance the formation of myotubes and activate mechanotransduction related genes. Also, it was found that the

synergistic properties helped to align and differentiate C2C12 myoblasts and thus, generate functional myofibers [127].

In another interesting study, a composite scaffold combining polymeric component, a mixture of gelatin and glycerol derivative, with resistant and flexible CNT has been developed in order to serve as cardiac tissue engineering construct. Using the electrospinning technique, hybrid nanofibers were produced and contractility of cardiomyocytes seeded on those scaffolds significantly improved with the inclusion of CNT. This formulation can be considered as a suitable platform, with correct mechanical and electrical properties, to use it as a graft for cardiac tissue constructs [128].

3.3 Control release of bioactive molecules

Not only biomaterials and cells are important in order to obtain efficient tissue repair or replacement, bioactive molecules that mimic the natural microenvironment and allow communication between cells also play a crucial role. Combining materials with suitable properties, cells and signaling biomolecules could be an effective strategy to achieve successful results in the field of tissue engineering [129,130].

Signaling biomolecules are generally growth factors, cytokines, chemoattractants or adhesion proteins that are released in their active forms locally with a sustained profile. Hence, these molecules largely influence cell behavior. Growth factors are defined as polypeptides that bind to specific receptors found on the surface of cells and, thereby, modulate cell proliferation, differentiation, migration, adhesion and gene expression due to activation of complex intracellular cascades [9]. Therefore, the use of growth factors accelerates the proliferation and differentiation of the implanted cells and helps to promote tissue regeneration. However, their chemical or enzymatic degradation and deactivation under physiological conditions occurs in a very short period of time. For that reason, to use these factors it is necessary to design and develop dosage forms that prolong the biological activity of proteins and target them to specific tissue. Certain spatiotemporal control allows both enhance the effectiveness of the regeneration process and prevent unwanted and potentially harmful side effects [131].

Hydrogels based on natural polymers such as gelatin are particularly promising materials in tissue engineering as they may have dual action, holding cells and, at the same time, encapsulating and releasing water-soluble compounds in a sustained mode. Thanks to the hydrophilic nature of gelatin, it can be used to coat the surface of drug delivery carriers, inhibit opsonization and improve water solubility [132].

As it has already been described above, gelatin can exhibit different charges and this makes it particularly interesting to complex with oppositely charged proteins. The kinetics release of bioactive molecules from gelatin-based systems depends on its degradation and

water uptake properties. This versatility allows choosing the best condition for achieving the desired release profile. This profile can be optimized and adjusted by changing gelatin source, molecular mass, crosslink degree and even developing composites with synthetic or natural polymers [20]. Hyaluronan [133,134], chitosan [135] and silk [136] are naturally derived polymers used to fabricate composite system with gelatin in order to enable biomimetic strategies for drug delivery. Among synthetic polymers polylactic-co-glycolic acid (PLGA) [137], oligopolyethylene glycol fumarate (OPF) [138] and polypropylene fumarate (PPF) [139] have been used with gelatin to produce suitable platforms for the delivery of bioactive molecules.

In recent years, multiple composite delivery systems have been designed to release different growth factors simultaneously from a single formulation. In this regard, gelatin seems to be an excellent candidate, since there are several dosage forms consisting of gelatin to deliver bioactive molecules, such as insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) [12,140].

4. GELATIN-BASED SYSTEMS

Over the last decades, gelatin has been used in the biomedical and pharmaceutical fields. Due to its versatility, this biomaterial has been used for the successful development of attenuated viral vaccines, for immunization against diseases such as diphtheria or rubella where gelatin acts as a stabilizer, for the production of hard and soft capsules or even as a component of plasma expanders formulations [18]. Besides, gelatin has been considered as an interesting candidate to elaborate formulations for advanced therapy, where cells and biologically active factors gain prominence.

One of the biggest advantages of this material is the ability to serve as material for various types of formulations with very different purposes in the field of tissue engineering. In this regard, gelatin nano and microparticles have been successfully developed to release both growth factors and cells. Gelatin-based three-dimensional (3D) scaffolds are widely used as carriers of cells and bioactive molecules to guide tissue regeneration. As a variation of these formulations, 3D formulations based on nanofibers fabricated by electrospinning are gaining interest because of the great advantages that show these nanometric fibers. Finally, gelatin is suitable for the development of *in situ* gelling hydrogels that can be administered by injection.

4.1 Particles

Gelatin particles have been used in recent years not only in the field of tissue engineering but also as a carrier system for many applications. In this regard, gelatin particles have been loaded with many types of drugs, such as anti-cancer drugs (methotrexate, cytarabine,

Table 1: Resume of gelatin-based composite systems examples

Improved property	Composite materials	Formulation type	Application	Ref.
Conductive property	Gelatin/PANI	Nanofibrous scaffold	Cardiac tissue engineering	[109]
	Gelatin/chitosan/PANI/graphene	Scaffold containing conductive nanoparticles	Peripheral nerve regeneration	[110]
	Gelatin/multi-walled CNT	Nanofibrous scaffold	Skeletal muscle tissue engineering	[127]
	Poly(glicerol sebacate)-gelatin/CNT	Nanofibrous scaffold	Cardiac tissue constructs	[128]
	Methacrylated gelatin/GO	Injectable hydrogel	Acute myocardial infarction therapy	[121]
	Gelatin methacryloyl/reduced GO	3D scaffold	Cardiac tissue engineering	[122]
	Gelatin/HA/GO	3D scaffold	Bone regeneration in orthopedics	[123]
Control of biomolecules release	Gelatin/hyaluronan/heparin	bFGF, VEGF, Ang-1, KGF, PDGF and TGF-β loaded hydrogel films	Promote angiogenesis in tissue replacement	[134]
	Gelatin/PVA/chitosan	Nanofibrous mats containing gelatin nanoparticles	Antibacterial activity in wound area	[135]
	Gelatin/silk/chitosan/glycerophosphate	Injectable hydrogel containing gelatin microspheres loaded with PDGF	Cartilage tissue engineering	[136]
	Gelatin/OPF	IGF-1 containing gelatin microparticles embedded in TGF-β releasing hydrogel	Osteochondral tissue regeneration	[138]
	Gelatin/PLGA/PPF	BMP-2 containing PLGA microspheres embedded in PPF/Gelatin hydrogel	Bone tissue engineering	[139]
Mechanical property	Gelatin/chitosan/alginate/HA	3D scaffold	Bone tissue engineering	[63]
	Gelatin/nanoHA	Nanostructured 3D scaffold	Bone tissue engineering	[64,66-68]
	Gelatin/TCP	3D scaffold with lumbrokinase	Bone tissue engineering	[70,72,74]
	Gelatin/TCP	Nanofibrous membrane	Bone tissue engineering	[71]
	Gelatin/PVA/BCP	Electrospun nanofibrous mat	Bone tissue engineering	[75]
	Gelatin/pectin/BCP	3D scaffold loaded with BMP-2 or VEGF	Bone tissue engineering	[76]
	Gelatin/hyaluronic acid /BCP	3D scaffold	Bone tissue engineering	[78]
	Gelatin/OCP	3D scaffold	Bone tissue engineering	[79-81]
	Gelatin/alginate/BCP/HA	3D scaffold	Bone tissue engineering	[82]
	Gelatin/PLA	Nanofibrous scaffold	Bone tissue engineering	[87,88,90]
	Gelatin/PLA	Nanofibrous tubular scaffold	Vascular tissue engineering	[89]
	Gelatin/PCL	Fibrous matrix	Vascular tissue engineering	[35]
	Gelatin/PCL/bone power	Electrospun fibers mat	Bone tissue engineering	[93]

PANI – polyaniline, CNT – carbon nanotubes, GO – graphene oxide, HA – hydroxyapatite, PVA – polyvinyl alcohol, OPF- oligopolyethylene glycol fumarate, PLGA – poly lactic glycoli acid, TCP – tricalcium phosphate, BCP – biphasic calcium phosphate, OCP – octacalcium phosphate, PLA – polylactic acid, PCL – polycaprolactone, bFGF – fibroblast growth factor b, VEGF – vascular endothelial growth factor, Ang-1 – angiopoietin 1, KGF – keratinocyte growth factor, PDGF - platelet derived growth factor, TGF-β – transforming growth factor beta , IGF-1 – insulin-like growth factor 1, BMP-2 – bone morphogenetic protein 2

doxorubicin…), didanosine, chloroquine, rifampicin, isoniazid or ibuprofen. In these cases, gelatin particles have got different functions. In some formulations, the main aim is to provide a sustained release of the drug, in some others, to reduce the toxic side effects, or to improve the pharmacokinetic profile and pharmacological activity of the drugs. Gelatin nanoparticles have also been used as a non-viral vector for gene therapy [141].

Besides, gelatin particles with different sizes, both micrometric and nanometric have been incorporated into formulations for regenerative medicine, as an important part for tissue engineering purposes.

4.1.1 Gelatin Nanoparticles

Nanotechnology is an area of great interest in various fields of science, with many potential applications in the biomedical and pharmaceutical fields [142]. Different nanosystems that can be administered by several routes have been designed, such as nanoparticles, nanofibers or liposomes. They are biologically stable and biocompatible, and can incorporate a myriad of bioactive molecules [143].

Polymeric nanoparticles are defined as colloidal solid carriers with a size in the range of 1 to 1000 nm from a natural or synthetic origin material. These particles can have an oily or aqueous inner core that it is covered by the polymer. In this group, various systems can be differentiated such as nanospheres or nanocapsules. Nanospheres are matrix system where the drug is physically and uniformly dispersed, while nanocapsules are vesicular systems [144]. Nanoparticles can serve as growth factors carriers system, where bioactive compounds can be loaded inside, promoting the enhancement of the efficacy of tissue engineering. The biological functions of encapsulated molecules can be improved by designing systems with controlled organization at the nanometer scale. With the nano-scale particles, it is possible to protect the bioactive molecule and increase their bioavailability due to the increased surface to volume ratio [145]. Nanoparticles are easy to design and prepare. In fact, in the literature, several methods for preparation of gelatin nanoparticles are described. Two-step desolvation, simple coacervation, solvent evaporation, microemulsion, nanoprecipitation and self-assembly are some of them. An extended report of all these techniques is described elsewhere, including discussion about their advantages and drawbacks [146].

Gelatin nanoparticles have multiple options to design modifications that make more advanced systems. In a work carried out recently, gelatin was modified with succinyl groups to convert it into a soluble substance at room temperature and crosslinked with aldehyde groups formed during the heparin oxidation process, avoiding the use of toxic crosslinkers such as glutaraldehyde. In addition, these nanoparticles served as nucleation sites for complexing calcium ions and thus, forming HA crystals, having as a result mineralized nanoparticles for potential use in tissue engineering of bone [147].

Poor biodegradability is one of the disadvantages that synthetic materials present for their use in biomedical applications. The use of gelatin in combination with these materials seems to reduce this drawback. In the case of gelatin nanoparticles incorporated into PCL nanofibers scaffold developed for bone tissue engineering purposes, the time to degrade synthetic PCL scaffolds was reduced. The degradation of nanoparticles increases the pore size of the structure and this promotes cell infiltration into the scaffold [148]. Advanced formulations containing gelatin nanoparticles have been increasingly exploited over the past few years, in order to develop systems for specific regenerative purposes, such as bone regeneration [148], cardiac repair [149] or even wound healing [150].

In a research work conducted recently, the aim was to stimulate endogenous cardiac repair in myocardial infarcted rat models. For this study an hybrid hydrogel containing two factors that have been shown to promote cardiomyocyte survival and proliferation was developed. 6-Bromoindirubin-3-oxime and IGF-1 were loaded in gelatin 180-255 nm nanoparticles to achieve a sustained co-release and improve cardiac function, promoting cardiomyocytes proliferation and revascularization of the infarcted area [149].

Achieving the control over the release profiles of the factors seems to be, precisely, the essential element required for an efficient regeneration. In this sense, multiple angiogenic growth factors have been studied for their use in wound healing. However, in order to mimic the physiological process followed in wound healing and skin reconstruction, the release kinetics of these biomolecules used in conjunction must be in accordance with their physiological functions. Some of them, for example, basic fibroblast growth factor, stimulate the recruitment of endothelial cells and some others have a greater role in the stabilization of new blood vessels. In order to approach these profiles, various methods to incorporate factors into a single system have been used. Direct incorporation of factors into electrospun nanofibers makes the release faster at the beginning; however, if the factors are within the gelatin nanoparticles, the liberation is slower and more sustained. This is what it was achieved with the addition of multiple angiogenic growth factors into electrospun composite nanofibers for chronic wound healing [150].

4.1.2 Microparticles

Until now, the most used method to fabricate gelatin microparticles has been the water-in-oil emulsion technique, followed by a crosslink phase. However, great efforts are being made to optimize the manufacturing process of these particles and, thus, get more advanced formulations with different surface (Figure 3A, 3B, 3C) [151]. In this regard, recently an innovative method to develop gelatin composite microspheres has been proposed. Spherical porous microspheres between 124 and 136 μm in size with rugged surface and nanofibrous structure were synthesized by emulsion coupled with thermally induced phase separation technique [152].

Gelatin microparticles have been developed to carry out cell therapy and, also, for delivering bioactive molecules in the tissue engineering field. In relation to the cell delivery methodology, several strategies have been completed to carry cells using gelatin microparticles, such as the attachment of cells on the surface of gelatin particles. In a study completed with progenitor cells attached outside of gelatin microparticles, researchers succeeded in increasing 10 times the number of attached cells in the ischemic myocardium compared to the injection of cells alone. This strategy has the advantage of administering cells in the cardiac tissue by the minimally invasive way using catheters and thus, avoiding open heart surgeries [153].

On the other hand, multiple cell types have been encapsulated within gelatin microparticles. W. Leung *et. al*. have designed an advanced hydrogel scaffolding system suitable for encapsulating non-anchorage-dependent cells that have propensity to form cell islets. This system combines gelatin microspheres loaded with chondrocytes and an alginate hydrogel. Microspheres are formed via the water-in-oil emulsion process but without any chemical treatment for the crosslinking. By increasing the temperature to 37 ˚C, the microparticles are dissolved and the cells are released from this particles remaining in the created pores. Gelatin microspheres play two roles in this cell delivery system; on the one hand, they are removable cell vehicles and, on the other hand, they act as porogens, creating cavities within the alginate hydrogel for better nutrient and waste diffusion [154].

Another interesting approach to increase the effectiveness of cell therapy, it is the use of empty gelatin microparticles embedded in a hydrogel containing cells. These microparticles are enzymatically digestible porogens and they provide cellular attachment site within the synthetic hydrogel. In fact, the long-term viability of MSCs is improved with the addition of gelatin particles with size between 50-100 μm, since these cells are anchorage-dependent. Also, gelatin microparticle loading modulates osteogenic differentiation and hydrogel mineralization *in vitro* [155,156].

In recent years, the use of gelatin microparticles on stem cells aggregates for the controlled delivery of growth factors to guide differentiation has gained interest. Gelatin microparticle incorporation within stem cell spheroids does not seem to essentially change cell organization. Nevertheless, the cellular microenvironment becomes more rigid and this is an advantage for promoting the stem cells differentiation toward lineages that are usually in stiffer tissues *in vivo,* such as bone [157]. Mesenchymal morphogenesis and stem cells differentiation can be promoted by incorporating degradable gelatin microparticles into embryonic stem cells aggregates. These methacrylate gelatin microspheres increase the expression of MMP, which facilitates the remodeling of the ECM and the control of cell differentiation [158].

Another strategy to control the degree of differentiation of pluripotent stem cells into the aggregates is to use gelatin microparticles loaded with specific factors in order to create a morphogen gradient within the aggregate. Spatially controlled differentiation within embryonic pluripotent stem cells spheroids was achieved using gelatin microparticle loaded with 125ng Bone Morphogenetic Protein-4 (BMP-4) per mg of microparticle. In fact, this type of signaling factors has difficulty to diffuse throughout the three-dimensional cellular aggregates, making it difficult to control that the differentiation is homogeneous [159].

In addition, gelatin microspheres have been widely used for their ability to deliver growth factors in different tissue engineering applications such as therapeutic angiogenesis, cartilage, bone and nerve tissue engineering and post infarction myocardial therapy [36]. As an example, microparticles loaded with glial cell-line derived neurotrophic factor were developed in order to promote sciatic nerve growth [160]. This formulation is a complex system based on gelatin, combined in various forms in a single construct. The system uses gelatin as material to fabricate microparticles but also as an hydrogel where particles are distributed (Figure 3D).

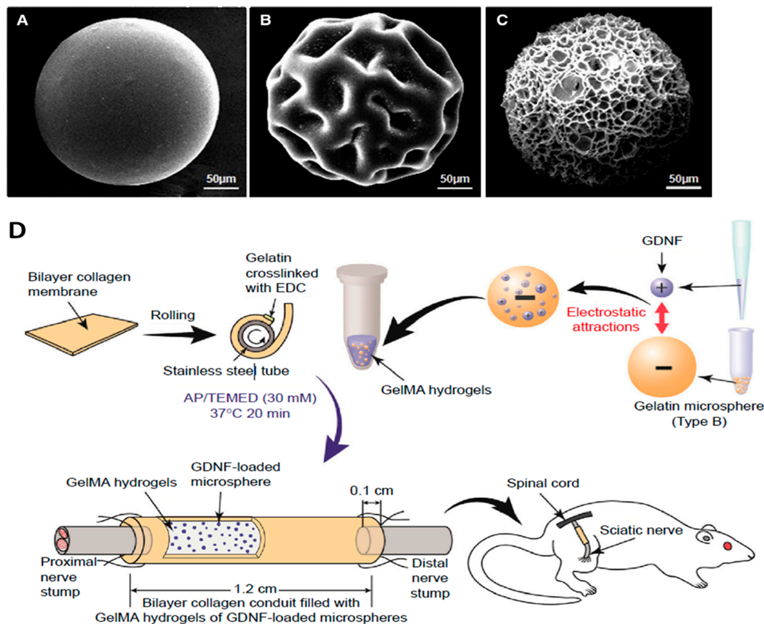

Figure 3. Microparticles of gelatin for tissue engineering. (A-C) SEM photomicrographs of microparticles of gelatin with different surface (bar=50 µm). (A) Smooth gelatin microparticle; (B) Pitted gelatin microparticle; (C) Multicavity gelatin microparticle. (D) Schematic picture of bilayer collagen conduit coated with gelatin-methacrylamide hydrogels containing glial cell-line derived neurotrophic factor (GDNF) loaded microspheres for promoting sciatic nerve growth in rats. A-C are adapted, with permission, from [151] and D adapted from [160].

Moreover, it has been found that by combining multiple growth factors in a single formulation, their therapeutic efficacy is enhanced [161]. Thanks to numerous studies, it has been shown that the combined use of IGF-1 and BMP-2 increases Alkaline Phosphatase (ALP) activity, which is extremely interesting in early osteoblastic differentiation. Sequential delivery of these two factors is achieved using gelatin microparticles loaded with IGF-1 encapsulated into a BMP-2 containing chitosan gel. Controlled release of these molecules is regulated by the crosslinking degree of the microparticles, the encapsulation of the particles into the gel and the interactions between proteins and carriers [162].

Furthermore, the release of the therapeutic agents from the gelatin microparticles is given by diffusion and also by the enzymatic biodegradation of the particles. It has been found that these microparticles degrade more rapidly in the post-ischemic brain of rats, due to the induction of gelatinase expression in that situation. Because of this, an increase in the neuroprotective effect of osteopontin was seen in cases where the protein was administered into gelatin microparticles since, in such cases, the release was rapid and sustained [163].

4.2 Scaffolds

Scaffolds are defined as implantable preformed 3D porous structures which support the regeneration process. Nowadays, it is a challenge to design and produce scaffolds that cover all necessary requirements mentioned above. However, gelatin is an ideal candidate to achieve these requirements, adding changes in its molecules and even designing hybrid matrices with different materials as described in the previous section. The incorporation of cells to 3D matrices can be done in several ways. For instance, cells can be cultivated onto the preformed structure. In a recent study, adipose-derived stem cells were distributed through the gelatin methacryloyl nerve guidance conduits scaffolds. These 3D cellularized scaffolds have been designed based on the general anatomical features of a rat sciatic nerve and fabricated by and indirect 3D printing technique (Figure. 4) [164]. Nevertheless, it is not easy to get uniformly cell-laden scaffolds because most of the cells remain on the surface. On the other hand, some researchers have developed a number of protocols that avoid the use of toxic compounds and severe conditions and thus, the incorporation of cells during the manufacturing process can be obtained [165].

In the case of scaffolds designed for drug delivery purposes, homogeneous dispersion of the biologically active agents throughout the structure is necessary in order to achieve sustained release of the growth factors and to avoid an initial burst effect [166].

For several years many techniques have been used to produce these 3D structures. Solvent casting/particle leaching, freeze-drying, phase separation, foam templating, fiber bonding, melt processing, electrospinning and rapid prototyping are the main processing methods employed to fabricate bioscaffolds [167]. Regarding the gelatin-based scaffolds, these are the

methods that have been used until now. Nevertheless, researchers have succeeded in introducing modifications [168] and even combinations [169,170] of these technologies. In this sense, scaffolds with discrete gradient in mechanical properties were developed in a recent work, by stacking mixtures of gelatin and collagen with different HA concentrations [171]. Amadori S. *et al*. used a similar strategy for designing hybrid structures containing differentiated layers for osteochondral replacement, by the method of overlap [172].

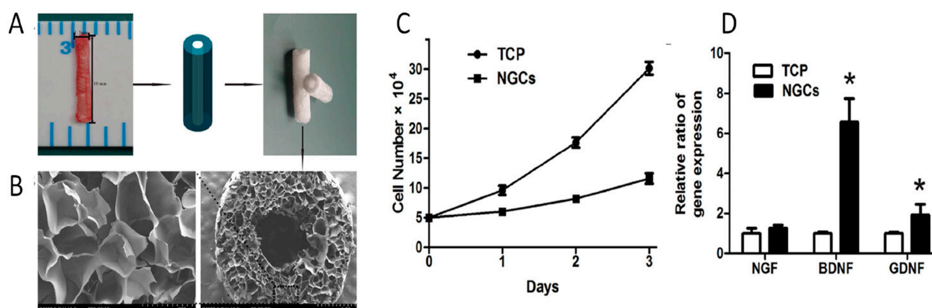

Figure 4. Cellularized gelatin based nerve guidance conduits (NGC) for peripheral nerve regeneration. (A) The measurement of diameters of the transected sciatic nerve for conduits design and fabrication. (B) SEM micrographs of nerve guidance conduits. (C) Analysis of the proliferation of adipose-derived stem cells (ASCs) on tissue culture polystyrene (TCP) and NGC after 1, 2, and 3 days of culture. (D) Gene expression of major neurotrophic factors of ASCs on the TCP and NGCs at 2 days post-seeding. Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF) and Glial cell-derived neurotrophic factor (GDNF). $*p<0.05$ for comparison with TCP. Adapted from [164].

In relation to the areas for applications of gelatin-based scaffolds, although great efforts have been made to produce these 3D formulations directed to different scopes of tissue engineering (wound healing [173], cartilage [174,175] and skin [176] among others), bone regeneration is the top field where more significant advances have been attained (Figure 5) [177]. A myriad of 3Dl structures based on gelatin and different composites have been successfully applied for bone tissue engineering.

This great effort includes *in vitro* and *in vivo* studies performed with different cell lines that have demonstrated the ability to differentiate into osteoblasts in gelatinous matrices. With reference to the cell types that have been seeded on gelatin structures for this aim, mouse osteoblastic MC3T3-E1 cell line, human primary osteoblasts, rabbit adipose MSCs and human dental pulp stem cells are some of them [178-182]. *In vitro* analyses include cell adhesion and proliferation assays, ALP activity quantification, examination of bone specific genes expression and immuno-histochemical staining.

Once the potential for bone regeneration of these scaffolds is demonstrated *in vitro,* there have been made many *in vivo* studies with different animal models in order to get evidence before taking the step towards clinical translation. Rat critical size calvarial defect experimental system is one of the most used in the evaluation of bone regeneration [76,183-185]. Nevertheless, the ability of gelatin-based matrices to promote ossification has also been tested in other animal models, such as rabbit ulnar critical size model [186] or X-ray-irradiated models. This last animal model wants to simulate the deterioration of the capacity of bone healing caused by X-ray irradiation used for the treatment of cancer. BMP-2 releasing gelatin-based scaffolds with autologous bone marrow got hold of structural regeneration at irradiated segmental bone defects [187].

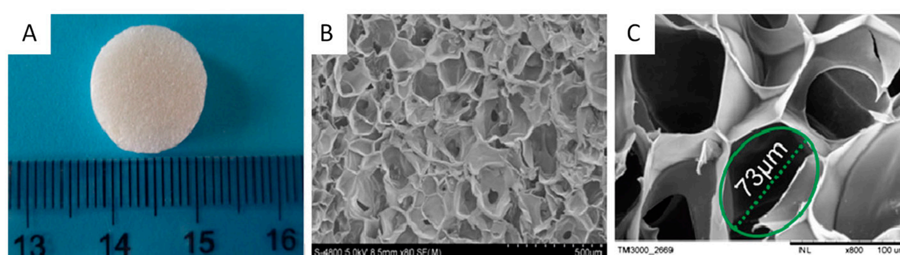

Figure 5. Chitosan/gelatin hydrogel scaffolds used as substitutes to mimic cartilage. (A) Gross morphology; (B) Surface topography and pore structure of the hybrid hydrogel scaffold; (C) A typical hole with a diameter which was measured at 73 μm on the surface of hybrid hydrogel scaffold. Adapted, with permission, from [175].

4.2.1 Electrospun gelatin-based scaffolds

Electrospun nanofibers structures are immensely versatile for an extended range of applications in several areas, such as the biomedical field. In recent years, the production of biomimetic architectures with nanoscale properties has gained interest to create 3D scaffolds with interwoven fibers to mimic the natural fibrous structure of the ECM. These bioscaffolds are highly porous structures with interconnected pores, fabricated usually by electrospinning technique. Electrospinning is a method for production of nano and submicron ultra-fine fibers from natural or synthetic origin polymers [188]. Electrospinning technique was introduced in the early 1930´s and since then there have been achieved many advances in the manufacturing process [189]. With this technology, fibers ranging from 50 nm to 10 μm can be fabricated [190]. The process can be performed with a wide variety of polymers, or even with the combination of several different polymers.

Regarding the variables to be determined during the process, there are many factors that can be classified into three groups: intrinsic polymer solutions properties, parameters related to the process and environmental parameters. All these variables will define the quality and

characteristics of the fibers and the resulting fibers network [191]. Producing morphologically uniform and compositionally homogeneous nanofibers is extremely important since it has an influence on cells adhesion and proliferation capacity [192].

In the case of gelatin, the conformation of the molecules in the polymer solution is of great importance. Formation of nanofibers by electrospinning can be performed only if gelatin adopts random coil conformation. There are two ways of creating gelatin nanofibers according to the nature of the solvent. When water is used to create the dissolution of gelatin, this solution must be heated above the sol-gel transition point, since at room temperature spinning of this formulation results impossible because of the high viscosity. In addition, the high surface tension of water solutions difficult the process due to the destabilization of polymer jets and the formation of droplets. Furthermore, the use of organic solvents to create the gelatin reservoir is an effective method to destabilize triple helix structure and allow electrospinning. Acidic solvent such as acetic acid [193] and formic acid, trifluoroethanol, dimethylsulfoxide, ethylenglycol and formamide have been used to improve electrospinnability of gelatin solutions [189]. The scaffolds of electrospun gelatin fibers have been used in applications such as wound healing [194], nerve [195], cartilage [196,197], bone [198,199], skin [200], cardiovascular [201] and ocular [202] tissue engineering.

In recent years, advanced nanofibrous scaffolds have been manufactured designing multilayer mats [203], core/shell structures [204,205], biologically active compounds-loaded nanofibers or structures with improved mechanical properties [191]. In a study performed with hybrid PCL/gelatin fibrous scaffolds, the sustained release of VEGF was possible by functionalizing gelatin fibers with heparin immobilization [206] (Figure 6). The development of scaffolds with larger pore areas and with higher porosity was achieved by combining electrospinning technique with gas foaming/salt leaching process. These scaffolds with a crater like structure provide higher proliferation and infiltration of human MSCs throughout the network [207].

4.3 *In situ* gelling formulations

In situ gelling systems can be considered as injectable grafts that have recently achieved promising results in the field of tissue engineering. These systems have several significant advantages over preformed scaffolds. On the one hand, they offer the possibility of minimally invasive administration into a target tissue, avoiding complicated surgeries and patient compliance would be better [208]. Moreover, because of the fluidity of these systems, they have demonstrated the ability to fill and replace temporarily complex and irregular shaped defects [209].

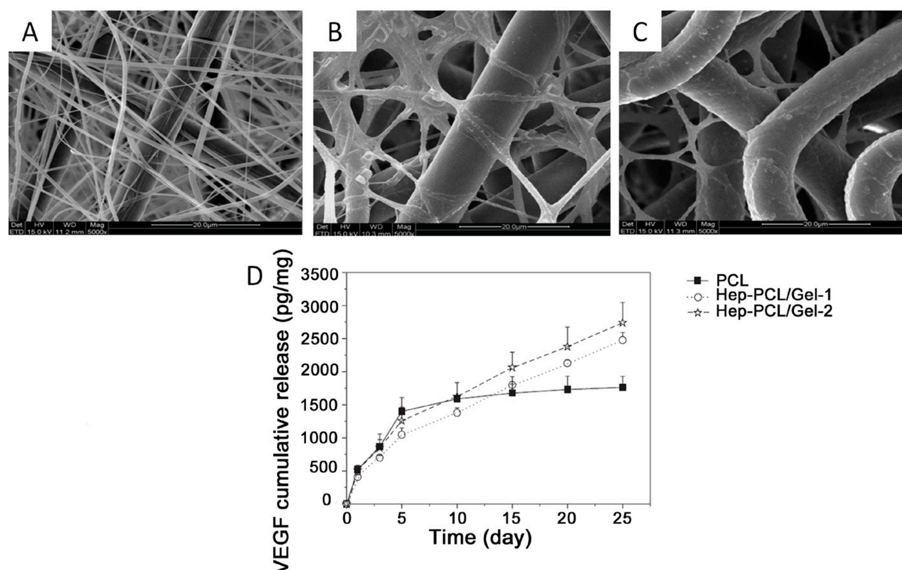

Figure 6. SEM images of structure characteristics and *in vitro* growth factor release profile of PCL/gelatin electrospun nanofibrous scaffolds. (A) PCL/gelatin nanofibers; (B) Heparinized PCL/gelatin nanofibers; (C) Heparinized PCL/gelatin degraded in phosphate buffer saline for 14 days. (D) *In vitro* release of VEGF from heparinized PCL/Gel scaffolds and PCL (n = 3). Adapted from [206].

In situ forming injectable hydrogels have been prepared using a variety of methodologies to get gelation physiological conditions. In any case, chemical reactions must be selective and fast to get effective designs. Injectable hydrogels can be prepared by chemical crosslinking, electrostatic interactions, self-assembly strategies, as well as stimuli-response methods [210].

Mechanically robust gelatin injectable hydrogels were prepared by a novel advanced "Host Guest Macromer" approach. Host Guest Macromer is the precursor for hydrogel fabrication, which is formed by complexation between aromatic residues of gelatin and the free diffusing photocrosslinkable acrylated β-cyclodextrins. Polymerization starts by UV irradiation and gelatin polymer chains are physically crosslinked. This strategy permits the fabrication of hydrogels with bioadhesive properties that have the ability to retain and release hydrophobic drugs and to support stem cell differentiation [211].

Sandeep T. *et. al* succeeded in developing click-crosslinked injectable gelatin hydrogels by introducing modifications on gelatin molecules. Hydrogels were formed within minutes and no external energy input, catalysts or initiators was required. Tetrazine and norbornene functional groups were attached to gelatin polymers in order to promote inverse electron demand Diels-Alder click reaction. As a result, they fabricated bioorthogonally crosslinked

gelatin-based hydrogels [212]. Recently, several researchers have used this methodology of click-crosslink to design gelatin-based *in situ* gelling grafts [213].

In situ gelling gelatin-based hydrogels have also been produced for both the delivery of cells [214] at the target tissue and the sustained release of growth factors, such as BMP-2 delivery in rat cranial defects [215]. Besides, injectable hydrogels offer a simple and effective procedure to research cell functional responses in a 3D environment, adjusting the *in vitro* assays to the reality that cells encounter in the tissues [216].

5. CONCLUSION

Full understanding of the tissue healing microenvironment that regulates tissue repair would help to make the translational step between academia and clinics propitious. In a certain part, the lack of success is due to the difficulty to recreate the complex signaling network carried out with multiples biological molecules. This review provides comprehensive overview of the potential of gelatin as biomaterial for its use in tissue repair and regeneration. Although significant progress has made already, much work lies ahead to move this biomaterial forward to routine clinical practice.

Until now, the introduction of changes in the structure of gelatin and the combination with various materials to create composite systems has proven to be a good strategy for formulating carriers for tissue engineering. Nevertheless, further investigations need to enhance bioactivity for specific tissues such as bone, cardiac tissue or nerve and to achieve maximum cell viability for long-term success. Future research direction that should be investigated about gelatin as a tissue-engineered material should focus on improving techniques to crosslink gelatin, with the aim of maintaining all the unique and ideal properties that this biomaterial has, without losing the biocompatibility that characterizes it. One of the major challenges to achieving translation is to scale up dimensions to clinically relevant sizes. Accordingly, future directions with gelatin should focalize on developing technologies to create functional and convenient size tissue substitutes.

Fortunately, there are reasons for optimism. Novel formulations and fabrication methods are likely to help broaden the catalog of gelatin-based applications. Designing operator-free technologies for fabrication together with the use of new technologies of additive manufacturing, or 3D bioprinting, may help to control the final properties of gelatin scaffolds and formulations. Advances in regulatory concerns related to safety and reproducibility will also be essential for future clinical applications in regenerative medicine. As a result of these and other advances, the safe and effective clinical implementation of gelatin-based products is expected to accelerate and expand.

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Recent advances in gelatin-based therapeutics

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ABSTRACT

Introduction: Biomaterials have provided a wide range of exciting opportunities in tissue engineering and regenerative medicine. Gelatin, a collagen-derived natural biopolymer, has been extensively used in regenerative medicine applications over the years, due to its cell-responsive properties and the capacity to deliver a wide range of biomolecules.

Areas covered: The most relevant properties of gelatin as biomaterial are presented together with its main therapeutic applications. The latter include drug delivery systems, tissue engineering approaches, potential uses as ink for 3D/4D Bioprinting and its relevance in organ-on-a-chip platforms.

Expert Opinion: Advances in polymer chemistry, mechanobiology, imaging technologies and 3D biofabrication techniques have expanded application of gelatin in multiple biomedical research ranging from bone and cartilage tissue engineering, to wound healing and anti-cancer therapy. Here, we highlight the latest advances in gelatin-based approaches within the fields of biomaterial-based drug delivery and tissue engineering together with some of the most relevant challenges and limitations.

Keywords: drug delivery, gelatin, regenerative medicine, tissue engineering .

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1. INTRODUCTION

Biomaterials include a broad class of substances that interface with biological entities and have over the decades provided a wide range of breakthrough opportunities in regenerative medicine. Our steadily increasing knowledge in biology, pharmacy, chemistry and material science, together with the interaction of these disciplines with one another, is rapidly expanding the application of biomaterials in the areas of controlled drug delivery, prosthetics and tissue engineering [1]. Among the many different types of biomaterials, bioactive polymers have advantages over their classic counterparts (i.e., static and non-stimulatory) as they are capable of playing an active role in drug delivery, control cell fate, regulate cell organoid formation and function for various drug testing applications, and even drive tissue repair and regeneration within the body [2,3].

The field of biomaterials has even more progressed due to a variety of prominent advancements in chemistry, cutting-edge imaging technologies, novel cell sources, avant-garde and optimized drug delivery platforms, increased knowledge on mechanobiology [4] (the process by which physical forces are converted into biochemical signals) and advances in various biofabrication processes such as three-dimensional (3D) bioprinting [5] and self-assembly technologies.

Gelatin is one of these particularly fruitful biomaterials, holding enormous promise for the above mentioned biomedical applications. This natural polymer derived from collagen, contains enjoys integrin binding cell adhesion peptides [6] and matrix metalloproteinase (MMP)-sensitive peptide sequences allowing cell-triggered degradation. Besides, one of the main features of this water-soluble protein is its thermo-responsive character, undergoing a reversible sol-gel transition when cooled upper its critical solution temperature (25-35 °C). Since gelatin provides a biologically active 3D microenvironment for regulating cell viability, growth and differentiation, it has attracted a great deal of interest nowadays either as scaffolds for tissue regeneration or carriers for controlled drug delivery. Currently there are several gelatin-based medical devices marketed by companies worldwide. For example, Gelita-SPON®, Cutanplast®, Gelfoam® and SurgiFoam® are absorbable gelatin sponges indicated to be used as hemostats in surgical procedures such as neurosurgeries, thoracic surgeries or ocular surgeries. More interestingly, gelatin containing allograft product has been approved by Food and Drug Administration for bone tissue engineering purposes. DBX® Strips is a flexible and bendable osteoinductive tape composed of demineralized bone matrix, gelatin and sodium hyaluronate, and it is indicated as a bone void filler. Moreover, a novel gelatin surgical implant, XEN® Gel Stent, has been developed to safely and effectively reduce the high eye pressure in refractory glaucoma cases. Regarding the commercialization of gelatin containing medicines, strict safety and quality requirements are demanded from the manufacturers involved in the production of gelatin for human use. The main objective

of this rigorous control is to completely avoid the risk of bovine spongiform encephalopathy associated with the use of animal origin raw materials. The pharmaceutical grade gelatin must comply not only with the current requirements for edible gelatin, but also with the provisions for medical products. In this regard, the European Pharmacopoeia lists specific requisite necessary for the manufacture of pharmaceutical grade gelatin.

In this review, we highlight the potential applications of gelatin in biomedicine and describe the corresponding advances in drug delivery and tissue engineering.

2. GELATIN-BASED BIOMATERIALS

Due to its cell-stimulatory properties, gelatin has been widely used in regenerative medicine and tissue engineering. Gelatin is a natural origin polymer, which holds several advantages over its precursor collagen. The low water-solubility of collagen under neutral conditions is one of the main limitations for biomedical purposes [7]. This drawback of collagen is can be overcome with the extraction process of gelatin. Another notable property of gelatin is its ability to create poly-ionic complexes with charged therapeutic compounds such as proteins, growth factors, nucleotides and polysaccharides [8], which in turn makes it ideal as a delivery vehicle for a broad range of biomolecules (Figure 1). In fact, gelatin exhibits depending on its extraction conditions either a net positive (IEP= 9, Type A gelatin) or net negative (IEP= 5, Type B gelatin) isoelectric point at pH 7.4, which allows sequestering oppositely charged proteins while maintaining its bioactivity. As a consequence, gelatin is a primary material in the fabrication of microcapsules and microspheres for drug delivery [9]. Furthermore, the source and the extraction conditions are key factors for obtaining gelatins with diverse physicochemical properties (melting temperature, gel modulus or viscosity), due to the differences in the amino acids proportions and the molecular weights of the resulting materials [10]. On the other hand, gelatin is easily functionalized to fabricate materials with tailor-made features, opening new therapeutic applications.

As an example, gelatin-based 3D microgels can be used to stimulate cell proliferation and differentiation of various encapsulated cells, such as stem cells, and can also improve the regenerative impact of injected cell-laden microbeads in lesion sites [11]. Over and above, these microgels can shield the cells from shear-force associated mortality during injection, and provide them with a milieu that enhances cell retention within the targeted site.

Crosslinked gelatin scaffolds can be used for 3D cell culturing. However, thermal gelation of gelatin typically results in frail and weak gels. To address this, conventional chemical procedures are applied to develop covalently crosslinked gelatin-based hydrogels [12]. One of the striking strategies proposed by several researchers has been the enzymatic crosslinking of gelatin systems, using various enzymes such as horseradish peroxidase [13]

or microbial transglutaminase [14]. These systems have been evaluated for different tissue engineering purposes, since versatile hydrogels with tunable gelation rate and final mechanical strength have been successfully developed. As an example, injectable enzymatically crosslinked gelatin-hydroxyphenylpropionic acid composite hydrogel have been evaluated both for cartilage [15] and brain [16] tissue reparation. Furthermore, photocrosslinkable gelatin methacrylate (GelMA) hydrogels have garnered great interest in various therapeutic applications, ranging from corneal tissue engineering [17], to peripheral nerve regeneration [18] and cartilage construct fabrication [19]. Furthermore, injectable covalently crosslinked gelatin hydrogels have been recently developed with the aid of pendant tetrazine or norbornene click chemistry pairs in modified polymers [20]. These gelatin polymers rapidly crosslink in combination and they start to degrade when injected *in vivo.* Moreover, they promote high cell viability and have the capacity to drive encapsulated cells into 3D elongated morphologies.

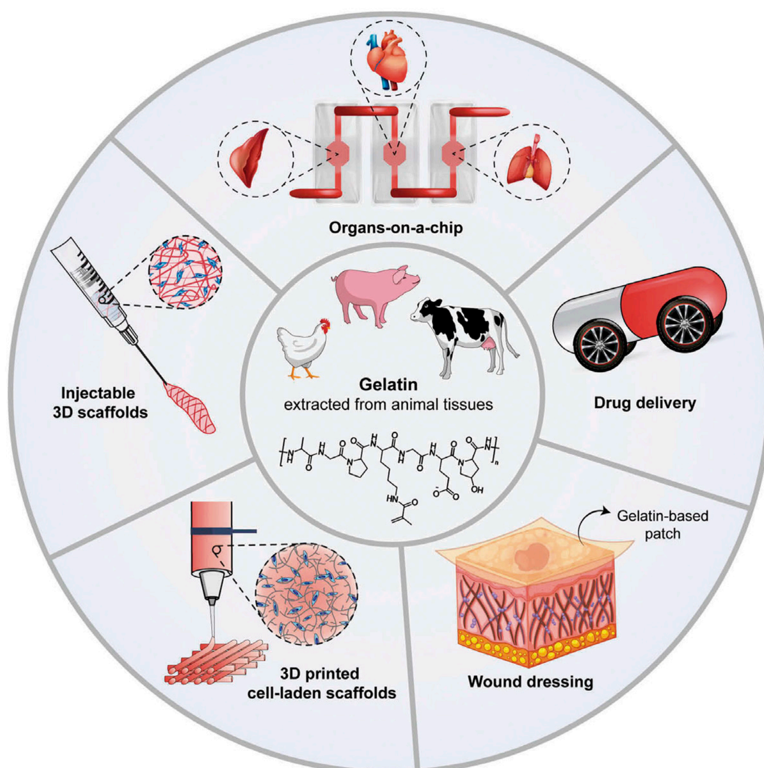

Figure 1. Some of the most promising approaches, being developed with gelatin as a primary biomaterial, including micro and nanoparticles for drug delivery, wound dressings, injectables, 3D scaffolds, bioinks and organs-on-a-chip technologies.

The ongoing progression in 3D bioprinting enables unprecedented control over spatial distribution of materials, cells and biomolecules and ultimately facilitate the fabrication of more native-like 3D tissue structures. In one noteworthy example, Jia W and colleagues designed a direct 3D multilayered coaxial extrusion printing strategy to develop highly perfusable and organized vessels. The blended bioink consisted of sodium alginate, gelatin methacrylate and 4-arm poly (ethylene glycol)-tetra-acrylate (PEGTA). This double crosslinked (covalently and ionically) system allowed the proliferation and propagation of encapsulated endothelial and mesenchymal stem cells inside the 3D printed scaffolds and ultimately facilitated the formation of native-like perfusable vessels [21].

3. PROGRESS IN DRUG DELIVERY

Gelatin-based biomaterials are of great interest in the design and fabrication of drug delivery systems, which provide controlled, sustained and/or targeted release of bioactive molecules, while enhancing their bioavailability and improving their therapeutic effects. This modern strategy is currently employed for several biomedical applications, ranging from tissue regeneration and wound healing to anti-cancer treatments and medical imaging. Wound healing is a highly complex dynamic process during which a damaged or injured tissue is repaired or replaced. Skin wounds are mostly due to trauma, surgery or burns; however, diseases such as diabetes may give rise to a prolonged healing time [22]. Gelatin-based dressings provide high protection capacity against infections and can accelerate the wound healing process, thanks to their tailorable mechanical and degradation properties. More than that, gelatin also enjoys favorable biocompatibility and contributes to a balanced hydrophobicity/hydrophilicity of the wound-dressing, resulting in a suitable release of biomolecules. As an interesting example, gelatin was combined with poly(ε-caprolactone) (PCL) to establish a scar-inhibiting electrospun fibrous scaffold, loaded with transforming growth factor β1 (TGF-β1) inhibitor (Figure 2) [23]. Through this approach, fibroblast over-proliferation was effectively inhibited *in vitro*, and scarring was successfully prevented *in vivo* during a wound-healing process in rabbit ear model. In another recent study, a conductive composite scaffold was fabricated based on PCL/gelatin nanofibers and silicate-based bioceramic particles [24]. In this system the nanofibrous microstructure of the scaffolds mimicked that of the extracellular matrix, and in combination with the sustained release of silicon ions from the silicate-based ceramic, a synergistic and beneficial effect in diabetic wound healing was obtained.

The release kinetics of bioactive molecules and drugs is highly dependent on degradation rate and water uptake of the gelatin networks. These properties represent a great opportunity for preventing multidrug-resistant bacterial infections, by releasing antibiotics in a controlled manner [25,26]. For instance, Lee and colleagues introduced gelatin-hydroxypropionic acid hydrogels, formed in the presence of bactericidal H_2O_2 as antimicrobial injectable or

sprayable dressings [26]. Other potential application of gelatin-based drug delivery systems is in cancer therapy, where improving drug bioavailability and targeted delivery have always been a major practical concern. For instance, the combined administration of dendritic cells (DCs) and oncolytic adenovirus in a tumor environment has a potent antineoplastic immune effect. However, the rapid inactivation of the drugs within the tumor site could reduce their effectiveness. To address this challenge, injectable and biodegradable scaffolds based on gelatin have been developed, enabling an extended delivery of both DCs and oncolytic adenovirus [27].

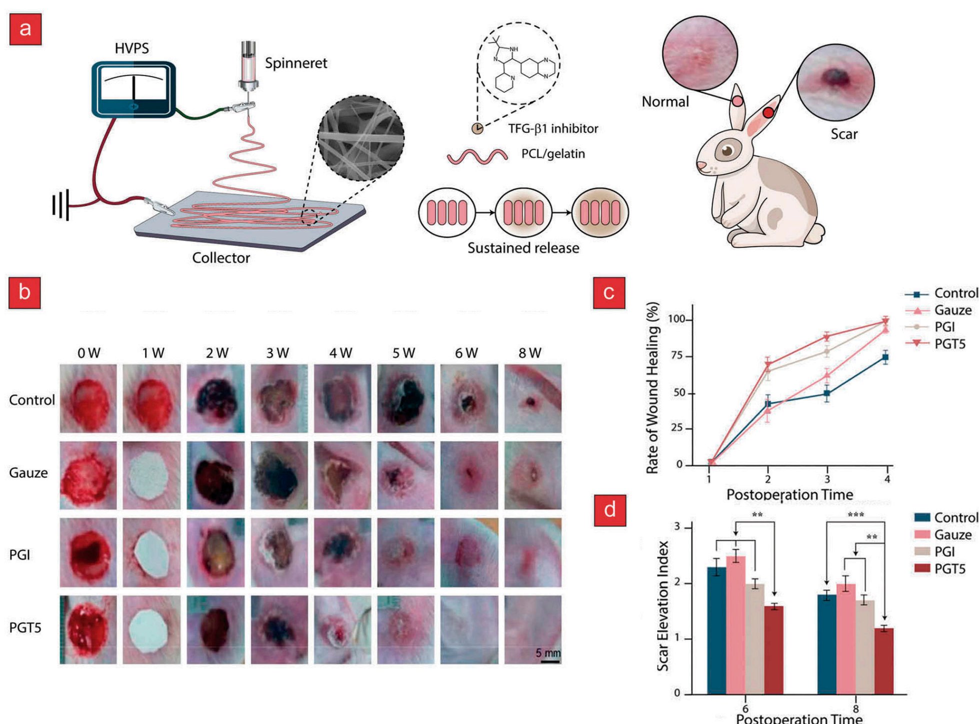

Figure 2. Hypertrophic scarring treatment with gelatin-based electrospun fibrous scaffolds. (A): Schematic illustration of the development of TGF-β1-inhibitor-loaded PCL/gelatin fibrous scaffolds. (B): Macroscopic evaluation of the wounds in different treatment groups (PGI: PCL/gelatin scaffold; PGT5: PCL/gelatin/TGF-β1 inhibitor scaffold). Only PGT5 group showed a perfect restructuration of the wound without irregularities. (C): The healed wound area of each group at different observation time points. (D): Elevation index of scars in each group, harvested at 6 and 8 weeks. Two asterisks represent a significant difference of $p<0.01$. Three asterisks represent a significant difference of $p<0.001$. TGF-β1: Transforming growth factor – β1; PCL: Polycaprolactone. Adapted with permission from Ref.[23] Copyright 2017 American Chemical Society.

Last but not least, gelatin-based micro and nanoparticles are emerging as promising flexible and powerful biomaterials for growth factor delivery. These characteristics are opening up new paths toward therapeutic alternatives in various medical fields such as cancer treatment, neuroprotection after post-ischemic brain injury [28] and immunization. As an example, Sabet S. and colleagues reported gelatin nanoparticles as a non-viral vaccine and gene delivery system for hepatitis C, which effectively transferred the nonstructural protein 2 gene into bacterial cells [29].

4. NEW ADVANCES IN TISSUE ENGINEERING

The overarching goal in tissue engineering is to develop artificial systems or structures that can recapitulate some of the most important functions of native tissues. However, this goal is challenging due to the extremely complex tissue architectures in the body, together with the many synergistic biological components within native tissues. The latest efforts in the field are currently directed towards combining different types of biomaterials to generate native-like synergistic effects, as well as providing the much-needed native-like tissue microarchitectures. Through this approach, several potential systems based on gelatin have been developed over the past decade. For instance, the combination of gelatin with hyaluronic acid was introduced as a biocomponent-based hydrogel to provide elasticity and promote vascularization of various target tissues [30].

Non-union bone fractures cause long-term incapacity and pain. Indeed, only in the United States around 8 million people suffers from bone injuries each year and conventional treatments are not effective in at least 10% of the cases. Several studies have recently emerged on new alternatives and bioactive agents for bone tissue engineering. In an intriguing approach, platelet loaded chitosan-gelatin composite hydrogel was applied to increased bone regeneration in bilateral critical-sized radial bone defect model in rats [31]. Furthermore, enhanced mRNA levels of alkaline phosphatase, as well as osteogenic (collagen type 1, osteocalcin, CD31 and run related transcription factor 2) and angiogenic (vascular endothelial growth factor) differentiation markers were also observed.

Even though gelatin-based scaffolds have not yet been commercialized for bone regeneration applications, the combination of gelatin with calcium phosphate ceramics and other synthetic polymers stand as promising options for clinical applications [32]. One of the biggest challenges here is to mold biomaterials into the similar complex architectures as those seen in bone defects. To overcome this limitation, several strategies have been studied including injectable systems [33] and 3D printed implantable structures [34], that facilitate fabrication of customized engineered scaffolds, that can perfectly adapt into bone defects (Figure 3).

In addition, new avenues are provided by tissue engineering approaches to facilitate the repair of the native cartilage tissue, which naturally presents limited capacity for spontaneous

repair. Over the last few years, several biomaterials including gelatin have been investigated and tested for cartilage tissue engineering applications. For example, the chondrogenesis of stem cells has been induced by co-culturing bone marrow stromal cell and chondrocytes in electrospun gelatin/PCL nanofibrous biomaterials [35]. In another approach, multipotent articular cartilage-resident chondroprogenitor cells were embedded in gelatin hydrogels to create cartilage-tissue regeneration [36]. These encapsulated cells presented a better profile in terms of neo cartilage production compared to differentiated chondrocytes. In addition, higher expression levels of the lubricating factor, PRG4, and lower expression levels of collagen type X hypertrophy marker confirmed the regenerative potential of these cells.

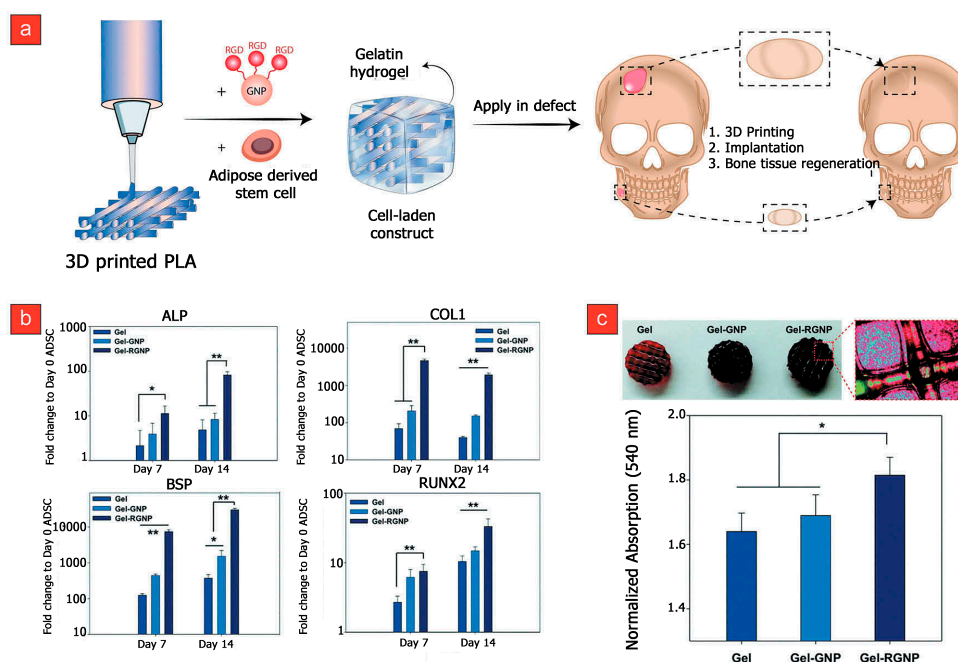

Figure 3. Bone tissue regeneration using a 3D printed microstructure incorporated with photo-curable gelatin hydrogel, functionalized with bioactive gold nanoparticles. (A): Schematic illustration of the therapeutic approach for personal bone tissue regeneration using 3D printing technology. (B): Gene expression levels of osteogenic differentiation markers of ADSCs cultured in the gelatin hydrogel (Gel), gelatin hydrogel functionalized with gold nanoparticles (Gel-GNP), and gelatin hydrogel functionalized with RGD-conjugated gold nanoparticles (Gel-RGNP). * indicates a significant difference of $p<0.05$. ** indicates a significant difference of $p<0.01$. (C): Optical images of calcium deposition staining from ADSCs culture in the three types of the hydrogel at 21 days. PLA: Polylactic acid; RGD: arginine-glycine-aspartate; ADSCs: human adipose-derived stem cells. Adapted from Ref. [34] with permission from The Royal Society of Chemistry.

5. LOOKING INTO THE FUTURE

Gelatin holds great promise for regenerative medicine, thanks to its biocompatibility and novel biomedical approaches such as cell-laden gelatin-based 3D tissue models are expected to be developed in near future to assist better understanding of diseases or drug development and screening. These 3D microphysiological structures could potentially complement or even replace current *in vivo* studies. As an example, gelatin microparticles have been combined with pancreatic cancer cells and fibroblasts to replicate the complexity of the pancreatic tumor microenvironment and to clarify the complex stroma-cancer inter-relationship [37]. In another intriguing approach, human cardiac microtissues have been bioengineered using cardiomyocytes derived from human induced pluripotent stem cells, encapsulated in gelatin hydrogels with tunable stiffness and degradation rates [38]. As a proof-of-concept Agrawal and colleagues designed a 3D skeletal muscle-on-a-chip platform with cell-laden gelatin hydrogel as a screening platform for drugs and toxics such as cardiotoxin [39], which can also be applied for preclinical drug discovery and development.

6. EXPERT OPINION

Gelatin is well-known biomaterial with exciting properties for protein and drug delivery and tissue repair and regeneration. Furthermore, it can be easily tailored to achieve different grades of mechanical stability and stiffness, being also an excellent candidate for controlling cell behavior, cell differentiation and even for cell transplantation. Gelatin can be used alone or combined with other biomaterials as bioink for 3D/4D bioprinting, opening new fields in the design and development of novel spatially customized anatomical structures in a personalized manner.

The use of gelatin however may see new horizons in the next few years. We envision that its properties may help to develop 2D and 3D nerve constructsbased on the mechanical properties and conductivity of novel gelatin-based composites. This progress may move forward its use in other cell-platforms such as organ-on-a-chip devices, that is, multi-channel 3D microfluidic cell culture chips by which it is possible to understand tissue and organ functions as well and test and screen a wide range of drugs. In addition, gelatin-based composites together with differentiated human stem cells may bring new hopes to heal spinal cord injuries and to limit the secondary damage.

Another interesting therapeutic window for gelatin relies on its potential use in transdermal delivery of therapeutics. By using biocompatible, biodegradable and bioresponsive gelatin-based microneedles, scientists are churning out new platforms for drug delivery across the skin in a safe and cost-efficient way. Last but not least, gelatin has been successfully combined and mixed with autologous growth factors derived from human plasma and platelets. Due its ability to sequester oppositely charged proteins while maintaining its bioactivity,

gelatin can provide additional properties to the naturally forming fibrin scaffold and thus be an aid in the therapeutic use of platelet rich plasma, an approach that is being used in multiple medical fields including dentistry, orthopedics, dermatology and ophthalmology.

Nevertheless, the use and applicability of this interesting material presents several challenges. The weak mechanical properties of gelatin may limit certain applications, especially those in which higher mechanical responses are demanding. To address this, both composite systems and chemical modifications are being explored. For example, aminoacidic structure of gelatin has been modified with the incorporation of diverse chemical motifs such as methacryloyl, catechol, phenol or epigallactocachecin gallate groups to increase its mechanical properties and durability.

In summary, gelatin provides a wide range of uses and applications in many different therapeutic fields. Progress in polymer chemistry, cell biology, mechanobiology, imaging technologies and 3D biofabrication techniques are expanding its value and potential in drug delivery and tissue repair and regeneration approaches.

7. CONCLUSION

Gelatin offers a broad spectrum of applications and possibilities ranging from micro/nanoparticle-based drug delivery to tissue repair and regeneration by means of 3D biomimetic scaffolds. We envision that the ongoing progression of research in this area will promote the future translation of gelatin-based biomaterials into the clinics.

8. DISCLOSURE STATEMENT

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Progress of Gelatin-based 3D Approaches for Bone Regeneration

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ABSTRACT

The current shortage of tissue and organ donor supply together with the often severe immune complications related to organ transplantation have fueled new scientific disciplines such as tissue engineering and regenerative medicine. The latter is an interdisciplinary field that applies biological and engineering principles to the design and development of technologies that promote regeneration to restore diseased and injured tissues, such as bone. There are a number of strategies to address this issue. Of particular relevance is the fabrication of biomaterial-based three-dimensional (3D) scaffolds, micro and nanoparticles as well as *in situ* forming hydrogels that are used to design temporary substitutes of the natural tissue that promotes its regeneration. This review article highlights the central role of gelatin for the design and fabrication of these formulations for bone tissue repair and regeneration. This review describes herein a state-of-the-art overview of gelatin formulations for their use in regenerative medicine strategies. Present challenges and future perspectives of these approaches are discussed in order to achieve safe and effective clinical implementation.

Keywords: gelatin, tissue engineering, regenerative medicine, bone, 3D scaffold, drug-delivery, cell-therapy.

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1. INTRODUCTION

It is widely known that tissue and organ transplantation is limited by donor shortage. The number of donors decreases every year whereas the number of the patients who die waiting for the organ transplantation increases. As it was recently reported by U.S. Department of Health & Human Services, more than 119,000 people entered on the national transplant waiting list but only 10,482 donors were recovered from January to August 2016 [1]. Even though transplantation is sometimes possible, the problem of graft rejection must be carefully considered as it directly affects organ and tissue-transplanted patients. In fact, patients will need to undergo immunosuppressive treatment, with related side effects, for all their life. In addition, the field of medicine often involves the use of biomedical devices and prosthesis that cannot replace the whole tissue alone with all its functions and that are unable to prevent a progressive deterioration of the health of the patient. Assuming this scenario and data indicating that percentage of patients >65 years old in developed countries will progressively increase in the next few decades, new medical solutions are urgently needed.

The fields of regenerative medicine and tissue engineering are a new field of medicine that aim to replace and restore injured and defective tissues or organs by using multifunctional constructs that may include biological and biomaterial-type structures [2,3]. One critical advance for these fields has been to combine the knowledge derived from different fields including molecular biology, biochemistry, biomaterial science, bioengineering, chemistry, medicine and pharmaceutical technology to design and fabricate a biological substitute designed *ad hoc* to replace or restore the damaged or lost tissue. Such a construct should in theory, unlike the classic biomedical device or implant used in reconstructive surgery, integrate itself with the surrounding tissues, restoring completely the damaged tissue function without the need of future pharmacological treatments [4].

To address this ambitious objective, it is of paramount importance to correctly combine 3D biomaterial-based scaffolds, biologically active signaling molecules and sometimes cells. In this particular review, we will pay attention to the fundamental role of the first of these three key ingredients, that is, the 3D structures or scaffolds. It is fairly recognized that the main function of 3D scaffolds is to provide a temporary support for the neotissue ingrowth from a chemical, physical and mechanical point of view. To accomplish this mission, it needs to fulfill highly specific criteria as it has been described elsewhere [5-7], including biocompatibility of the primary materials and the 3D construct, mechanical properties, suitable surface properties optimized for the potential attachment, migration, proliferation and differentiation of cell phenotypes, suitable 3D shape and architecture with an interconnected pore network, retention capacity for the biological cues and biodegradability with a controllable degradation rate that matches the cell/tissue in- growth and maturation and last but not least easy and efficient reproducibility. Some of these desirable properties are summarized in Table 1.

Table 1: Desirable properties of biomaterials for bone tissue engineering.

Properties	Description of the property
Biocompatibility	The material must perform with an appropriate host response in bone tissue regeneration. It is the ability to be in harmony with tissues without causing harmful changes.
Biodegradability	The material should ideally degrade without any non-native products remaining. The rate of degradation must be adjusted to the process of tissue regeneration allowing regenerated tissues to organize into the desired 3D structure.
Low toxicity and inflammatory response	The material must not have toxic effects on osteoblasts or other cells of the bone tissue. Additionally, the material must not be toxic systemically and should not possess toxic degradation residues.
Porosity and pore interconnection	High and interconnected porosity is essential for 3D scaffolds designed for bone tissue engineering. Pore size, pore volume, pore size distribution, pore shape and pore wall roughness will also define the ability of cell ingrowth and uniform cell distribution. All these parameters will have repercussion in the capacity of neovascularization of the matrix.
Biomechanical stability	The mechanical properties (elastic modulus, tensile strength, fracture toughness, fatigue and elongation percentage) should be similar to those of the bone to be replaced or regenerated.
Bioactive compounds protection and sustained delivery	Ideally, the biomaterial should be capable of sustained and controlled release of potentially therapeutic agents, in order to achieve adequate concentrations locally of growth factors that promote bone regeneration.
Processability	It is desirable that the material can be processed easily to design a variety of configurations and formulations such as 3D scaffolds, nanometric and micrometric particles or injectable formulations.

In the particular case of bone regeneration, although bone shows self-regeneration properties in some particular injuries, it is one of the most frequently transplanted tissues [8]. Bone grafting represents the second most common tissue transplantation procedure with over 2.2 million procedures worldwide annually in just two medical fields: dentistry and orthopedic surgery [9]. As discussed previously, the increase in the elderly population will lead to an increasingly frail population at greater risk of bone fracture, especially due to diseases such as osteoarthritis and osteoporosis, with a tremendous socio-economic burden on world healthcare systems [10].

A wide number of materials, technologies and approaches are being explored as novel bone tissue regeneration alternatives. Nowadays, there are several collagens/gelatin based products available in the market for bone tissue engineering purposes such as, DBX® or RegenOss® [11]. In this review, we aim to discuss recent progress derived from the use of gelatin as biomaterials and particularly gelatin-based 3D scaffolds as constructs. The most relevant results obtained so far together with the limiting challenges will be reviewed.

2. GELATIN AS BIOMATERIAL

Gelatin is a natural origin water-soluble protein derived from the hydrolytic process of collagen, where triple helix of collagen is broken up and single strand macromolecules are

obtained. This biomaterial has an amphoteric behavior, because of the presence of alkaline and acidic amino acids functional groups.

Several sources have been used for many years to extract gelatin. In this regard, gelatins of mammalian origin have been the most frequently used materials for regenerative purposes. These gelatins have been chiefly obtained using porcine and bovine skins and bones [12]. However, these materials do not meet the optimal characteristics that may require a material to be employed in tissue engineering. In fact, with the use of these materials, there is a risk of transmission of pathogenic vectors such as prions and the development of bovine spongiform encephalopathy. For this reason, great efforts have been made to obtain gelatins of other alternative origins to avoid this type of risks. Gelatins from warm and cold water fish skins, bones and fins have been produced using a number of disparate approaches. Fish derived gelatin has a significantly higher viscosity and lower melting temperature and thermal stability. These differences in properties are because of the differences in both the amino acids compositions and the corresponding sequence [13].

Another novel strategy has been the development of recombinant gelatins to overcome the disadvantages and improve the properties of materials derived from different animal tissues. Gelatins with accurate molecular weight and the isoelectric point can be produced using various expression systems such as *Pichia pastoris* or *Hansenula polymorpha* yeast, *Escherichia coli* and transgenic mice or tobacco plant. With this technology, the reproducibility between batches is significantly increased, thus improving control over the properties of the material. There are two different techniques for producing recombinant gelatin. One is the synthesis of recombinant collagen to later purify and denature with or without chain fragmentation. The other is to directly produce specific chains of gelatin [14].

Gelatins are polymers of a mixture of amino acids moieties joined by peptide bonds ranging in molecular weight between 15,000 and 400,000 Daltons. Regarding the structure of this biomaterial, more than twenty amino acids in variable proportions comprise its primary structure. Gelatin molecules are composed of repeating sequences of glycine-X-Y triplets, where proline for X and hydroxyproline for Y positions are the most common amino acids [15]. During the hydrolysis collagen tertiary structure triple helix is broken down into single chains (α-chains), covalently crosslinked double α-chains (β-chains) and triple α chains species (γ-chains) [16]. The length of the polypeptide chains and the proportion of each type of chains is different depending on which is the raw material, the pretreatment method used, process duration and some processing parameter such as temperature or pH. There are three phases in the process of producing gelatin from collagen. The first step is the pretreatment of the selected raw material, the second one is the extraction itself, and finally the purification and drying of the material obtained are necessary [17]. The method used for the pretreatment of the material will determine the electrostatic charge of gelatin and thus, the type of gelatin

to be obtained, which can be of two types. If the collagen hydrolytic process is exerted under acidic conditions using sulfuric acid or hydrochloric acid, the gelatin produced will be Type A and its isoelectric point is around 9.00. In contrast, if the pretreatment chose is alkaline, the gelatin obtained will be Type B and will present the isoelectric point around 5.00. The isoelectric point of a peptide is defined as pH value at which the net electric charge of the molecule is zero. When the pH value of the solution is lower than the isoelectric point, the peptides are positively charged, whereas in higher pH values conditions they present negative net charge. This ability to produce gelatins of different isoelectric point modifying parameters of the process is one of the greatest advantages that this biomaterial presents for its use in the biomedical field [18]. Therefore, gelatin can be used as a carrier for therapeutic agents charged both positively and negatively by polyion complexation. Acidic Type B gelatin is suitable for the sustained release of basic molecules under physiological conditions, while basic Type A gelatin should be used to carry acidic proteins *in vivo.*

Gelatin is natural origin biocompatible and non-cytotoxic material and it shows low immunogenicity compared to the native collagen. This polymer is considered as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) [19] and it is biodegradable because of the presence of sensitive sites for enzymatic degradation by matrix metalloproteinases (MMP). In addition, it has been found that the byproducts resulted in the enzymatic degradation are themselves biocompatible [20]. Furthermore, gelatin present in its structure sequences of arginine-glycine-aspartic acid (RGD). This fragment is the specific recognition site of integrins and it is involved in the regulation of interactions between cells and between cells and extracellular matrix (ECM). It is considered as a biomimetic peptide that promotes cell adhesion, with the ability to prevent the cell from apoptosis and accelerate tissue regeneration [21,22].

Regarding physicochemical properties of gelatin, one of the most important properties is the potential to form a thermally reversible network in water. It presents conformational transition below sol-gel transition point at about 30 °C. In the gelation process, locally ordered regions are formed which are joined by non-specific bonds, such as electrostatic, hydrophobic or hydrogen bonds. When the gel is heated, these intermolecular interactions are broken, therefore obtaining the thermo-reversibility, the unique property of gelatin [23].

It has been shown that there are different factors that define the properties of the gelatin dispersion. The rigidity of the gel is determined on the one hand by the temperature, but on the other hand, the concentration, bloom strength, pH and the presence of any additive in the dispersion can modify its stiffness. The bloom strength is the intrinsic force of the physical gel that it is formed upon cooling. This value is determined by the structure of the gelatin itself together with the molecular weight. Temperature plays an important role in defining the gels

final properties since the viscosity and gel strength values decrease with increasing temperature above 40 °C [24].

Crosslinking of the gelatin strands is necessary to improve the thermal and mechanical stability under physiological conditions, in order to use these formulations in medical applications, especially in tissue engineering field [25]. Many studies have been carried out over the past years to determine how the agent or method used to crosslink the gelatin network influences the physical, mechanical and, in particular, cytotoxic properties of the hydrogel.

The introduction of previous modifications in the gelatin molecule allows improving the control over the crosslinking process and therefore the final properties of the hydrogel. The incorporation of functional groups such as norborene, feluric acid, succinyl, acrylamide or even methacryloyl has allowed the design of formulations with unique features [26-29].

The crosslinking of gelatin hydrogels can be done by physical or chemical processes. The crosslinking of gelatin strands by physical techniques can be achieved using microwave energy [30], dehydrothermal treatment [31,32] and ultraviolet radiation [33]. These processes do not employ any chemical agent that may be biologically toxic, but control over crosslink density and process efficiency is often low [34]. In contrast, agents which are incorporated into the gelatin molecules or agents which have the ability to activate functional groups of the gelatin molecules are used to get chemical crosslinking. Aldehydes, polyepoxides and isocyanates form chemical bonds with gelatin molecules, which can be released as reactive and toxic agents in the degradation process [35-37].

In this sense, aldehydes have traditionally been the most frequently used agents to crosslink gelatin. Nevertheless, it has been shown that the products generated as a result of the degradation process can have toxic effects on cells, exhibit immunogenicity and inflammatory response. Therefore, in recent years the use of other alternative agents has been investigated, in order to reduce the undesirable toxic effects.

One of the crosslinking agents that has gained more interest is genipin. Genipin is a biocompatible agent extracted from the fruits of the *Gardenia jasminoides* plant that is being used to crosslink different materials in biomedical applications. This agent has been shown to have high crosslink efficiency and 5000-10000 times lower cytotoxic effect than glutaraldehyde [38,39]. Another potential strategy to achieve stability of gelatin under physiological conditions is to crosslink its strands enzymatically. Enzymatic crosslinking with tyrosinases, transferases and peroxidases is a strategy that is gaining interest for hydrogels in tissue engineering. These reactions occur in aqueous media, neutral pH, moderate temperatures and it is possible to achieve specificity towards the substrate [40,41]. Mushroom tyrosinase [42] and microbial transglutaminase (mTG) [43] are two of the enzymes that have been used to crosslink gelatin formulations.

3. GELATIN-BASED SCAFFOLDS

3D scaffolds are temporary artificial extracellular matrices that are used to deliver cells, drugs and genes into the body. One of the principal functions of scaffolds is to guide cells growth. In fact, these matrices play a crucial role in cell adhesion, differentiation, proliferation and new tissue formation in three dimensions. Preformed scaffolds are substitutes that can be implanted using surgical techniques and which are classified into typical 3D porous matrices or nanofibrous matrices depending on the methodology used for their fabrication.

Over the past few years, many attempts have been made to develop gelatin based 3D scaffolds for different purposes including skin regeneration [44], wound healing [45] or even nerve and cardiac tissue engineering. However, bone tissue engineering is likely to be the field in which more significant advances have been made so far. A myriad of 3D gelatin based prototypes and diverse composites have been designed for osseous tissues regeneration.

3D scaffolds can be manufactured using different production techniques: solvent casting/particle leaching, freeze-drying, phase separation, foam templating, fiber bonding, melts processing, electrospinning or rapid prototyping [46]. Although gelatin scaffolds have been produced largely following these protocols until now, current strategies are focused on the development new protocols to improve and to optimize the final properties of the prototypes [47-49]. For instance, Jelen C. *et al.* showed that stacking gelatin mixtures with different hydroxyapatite (HA) concentrations, discrete functionally graded scaffolds have been successfully prepared. With this new feature, they wanted to mimic the gradient existing in the structure of the bone in terms of mechanical properties and porosity [50].

When designing prototypes intended to be used in tissues that present challenging mechanical conditions as in the case of bone, it is necessary to take into account the high water content of the hydrogels. As a consequence, it is well established that the mechanical strength of such formulations is usually limited. To overcome these drawbacks the gelatin can be combined with diverse compounds to further improve the mechanical properties of gelatin-based composite scaffolds [51]. For example, calcium phosphate ceramics are especially interesting when designing formulations for bone tissue engineering not only to improve the necessary mechanical properties but also because they provide convenient osteogenic characteristics. Most of them have been shown to have osteoconductive properties (support of osteoblasts adhesion and proliferation) whereas some of them have been attributed to osteoinductive properties (capacity to stimulate the formation of new bone by recruiting progenitor cells or inducing differentiation into osteoblastic lineages) [52,53]. There are currently many examples of systems designed following this strategy. Gelatin composites with HA [54-57], tricalcium phosphate [58-60], biphasic calcium phosphate [61,62] and octacalcium phosphate [63] have been proposed as appropriate constructs to be used in bone tissue engineering.

Complementarily and as an important part in the development of gelatinous matrices with osteogenic features, the study of the interaction of different cell types both *in vitro* and *in vivo* has opened the way to optimism. In many cases, 3D scaffolds made primarily with gelatin have been shown to be able to differentiate different cell types into specific cells of the bone tissue. The most characteristic *in vitro* studies for evaluation of these properties are cell adhesion and proliferation, quantification of alkaline phosphatase (ALP) activity, evaluation of the expression of specific bone genes and immunohistochemical staining. Mouse osteoblastic MC3T3-E1 cell line [64], human primary osteoblasts [65], human mesenchymal stem cells (hMSCs) [66] and human dental pulp stem cells [67] are only some of the cell types that have achieved satisfactory results.

However, undoubtedly, for the evaluation of the real ability of these scaffolds to promote bone regeneration, *in vivo* studies performed on different animal models are extremely important. The rat critical size calvarial defect model is one of the most used methods so far [68-70]. In this sense, gelatin scaffolds have been recently evaluated in rabbit ulnar critical size model [71], X-ray irradiated models [72], rat distal femoral condyle defect model [73] and rat tibial bone defect model [74].

3.1 Bioprinting of gelatin-based scaffolds

Hopefully, the development of the 3D bioprinting technology has provided currently tools for achieving greater precision in the structural and mechanical properties of artificial scaffolds. This technology presents potentials in reproducible fabrication of prototypes with high accuracy for tissue engineering [75]. This technique consists of a computer-aided layer-by-layer deposition approach where living cells and other biological agents are stacked and assembled using biomaterials as a bioink vehicle for fabrication of living tissue and organ analogs for tissue engineering and regenerative medicine [76]. Although it is in its early stages, bioprinting strategies have become one of the most promising and advancing manufacturing methods. The principal advantages of bioprinting include a high resolution on cell deposition, cell distribution accuracy, cost-effectiveness and scalability [77]. In recent researches, it has been shown that the use of gelatin for the development of suitable bioinks provides interesting characteristics to cover the necessary properties that a bioink should have: biocompatibility, biodegradability, printability, crosslinkability and mechanical properties. More and more research groups work on studying the potential that this material could have for the development of these types of formulations [78-82].

For instance, Figure 1 shows the diagram of the bioprinting process of cell-laden scaffolds carried out with gelatin methacrylamide hydrogel dispersions. The equipment has a temperature control system and the crosslinking process of the gelatin is made using a UV

light source. The printer has the ability to tune the microstructure of the matrices in the micrometer scale, thus adjusting the pore size [78].

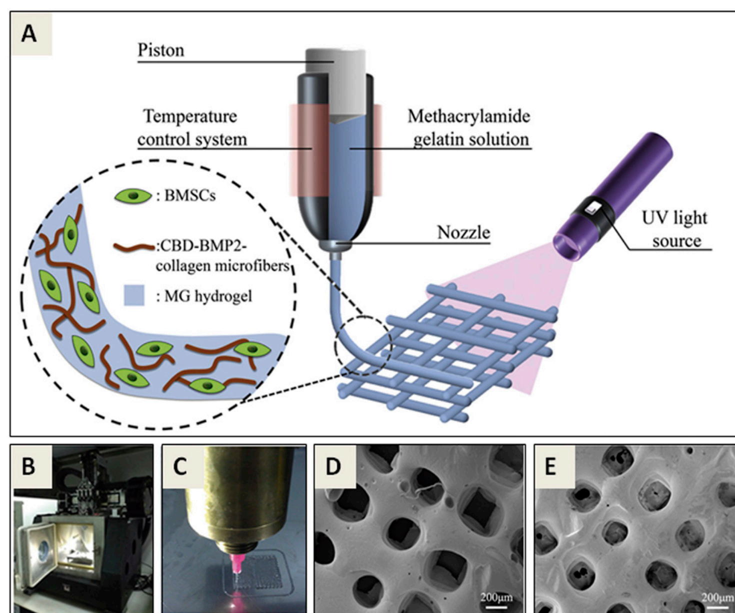


Figure 1. Fabrication of 3D scaffolds using customized 3D printer. (A) Schematic picture of the printing and photo-crosslinking process. (B) 3D printer equipped with refrigeration. (C) Printing nozzle. (D, E) Observation of printed methacrylamide gelatin scaffolds by SEM. (D) Scaffolds with a pore size of 363 ± 60 μm and (E) 282 ± 32 μm, scale bar = 200 μm. Reprinted from [78].

3.2 Electrospun gelatin-based scaffolds

In the last years, the development of systems based on electrospun nanofibers is immensely explored by researchers, due to the versatility that they show to be able to use in different biomedical fields, for example in the tissue engineering. Electrospinning technique is a production method that is useful to generate fibers ranging in diameter from 50 nm to 10 μm from both synthetic and natural origin materials. It is considered a simple technique where several parameters define the morphology and size of the fibers obtained. Through this methodology, biomimetic architectures with nanoscale characteristics and interwoven fibers that mimic the natural structure of the ECM have been successfully developed [83]. A wide variety of polymers and their simultaneous combinations have been used to make fibers. It is possible to obtain fibers composed of several polymers by mixing the dispersions in the same syringe, doing the process by two syringes placed side by side or even layering the dispersions in succession [84]. There are several factors that affect the final properties of the fibers. These variations

in the process allow manufacturing fiber mats with different properties, adapting to the desired use. The factors can be classified into three general groups: First, the intrinsic polymer dispersions properties (molecular weight, concentration, solvent type, viscosity…), second parameters directly related to the process (applied voltage, nozzle-to-collector distance, nozzle diameter…) and finally the environmental parameters (temperature, humidity…) [85].

In order to develop gelatin fibers by this technique, the state of the conformation of the gelatin molecules in the solvent is very important. In fact, it is possible to create such fibers only in cases where gelatin adopts random coil conformation. When the gelatin is dissolved in water, it is necessary to heat the mixture above the sol-gel transition point to reduce viscosity and be able to spinning. Alternatively, the use of organic solvents allows destabilizing the triple helix of the gelatin structure and thus it is possible to form gelatin-based electrospun nanofibers. In this regard, formamide, dimethylsulfoxide, acetic acid, formic acid, trifluoroethanol, and ethyleneglycol have been used to improve the electrospinnability of gelatin [86].

In order to achieve specific bioactivity towards bone applications, the incorporation of calcium phosphates and bioactive glasses seems to obtain satisfactory results [87-89]. As can be seen in Figure 2, the incorporation of biphasic calcium phosphate nanoparticles into polyvinyl alcohol and gelatin electrospun fibers forms a potentially advantageous material for attaining bone repair abilities *in vitro* and *in vivo*. The inclusion of the nanoparticles in the fibers increases the diameter of the fibers, the tensile strength, the adhesion and proliferation of human osteoblast-like MG-63 cells derived from human osteosarcoma as well as the expression of proteins related to bone formation. In addition, in the *in vivo* studies carried out on rat calvaria defects increased bone formation was observed at 2 and 4 weeks [90].

The use of biocompatible conductive polymers in order to deliver locally electrical stimuli is another strategy employed in formulations designed for bone tissue engineering. Piezoelectricity is one of the intrinsic electrical properties of the bone, which was discovered in 1950 [91]. This property has an extensive effect on the control of proliferation of osteoblasts. For this reason, in the research works carried out in recent years, it has been clearly stated that electrical and electromagnetic stimulation has a progressive influence on the treatment of bone healing, modifying activities of the osteoblasts [92]. More and more gelatin based matrices fabricated with electrospinning technique include in their designs conductive specific polymers, such as aniline [93]. In fact, recent data suggest that this incorporation not only improves the structural properties of the scaffolds but also increases bone healing by electrical stimulation [94].

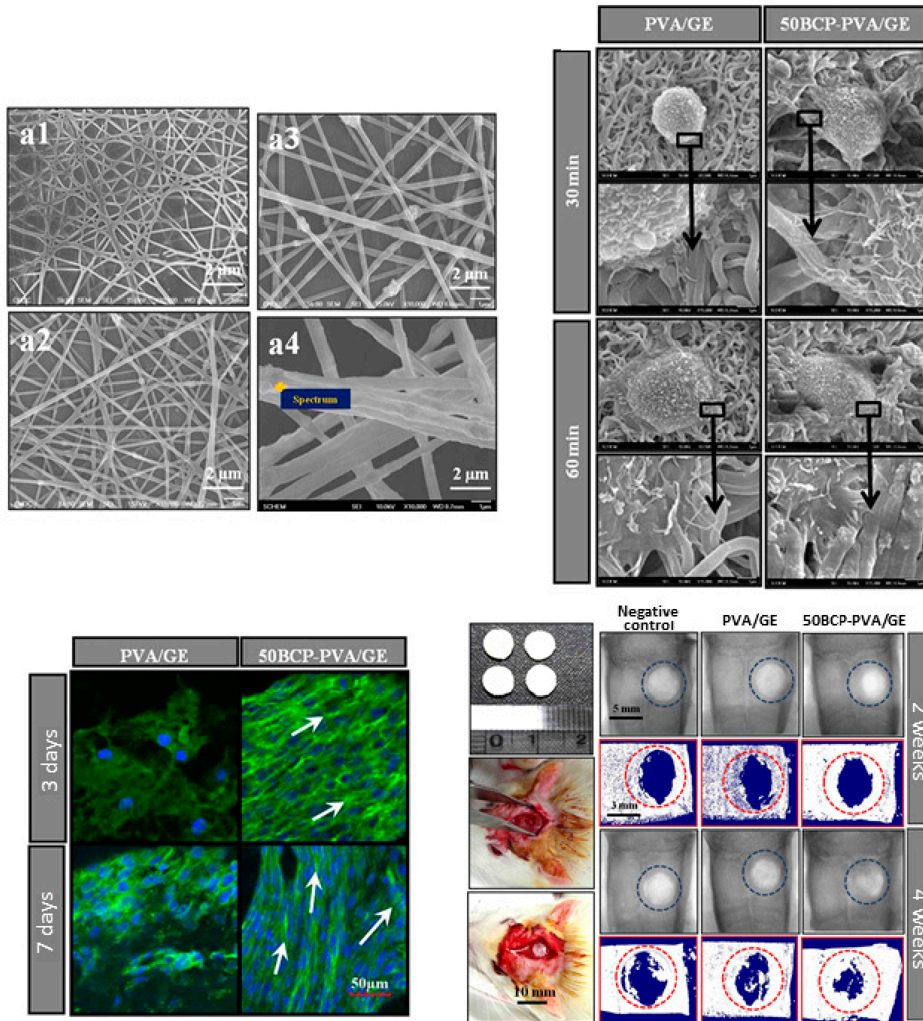


Figure 2. Biodegradable functional nanofibers of polyvinyl alcohol/Gelatin (PVA/GE) containing nanoparticles of biphasic calcium phosphate (BCP) for bone regeneration. (A) Morphology observed by SEM of (a1) PVA/GE fibers, (a2) 20%BCP-PVA/GE fibers, (a3) 40%BCP-PVA/GE fibers and (a4) 50%BCP-PVA/GE fibers. (B) Human osteoblast-like MG-63 cells attachment on electrospun membranes after 30 and 60 min seeding observed by SEM. (C) Confocal images of osteoblasts on electrospun membranes 3 and 7 days after incubation. (D) Micro-CT images and 3D reconstructed images of negative control and prototypes membranes implanted on a rat calvarial bone defect. Adapted, with permission from [90].

4. OTHER GELATIN APPROACHES

As mentioned above, one of the most interesting advantages of this material is the ability to use it to set down disparate formulations types. The easy handling and processability make it an ideal candidate to design diverse 3D efficient approaches for hard tissues regeneration purposes. Gelatin has been used in recent years as a material to create particles of nanometric and micrometric size as well as formulations that gel *in situ*, so that they can be injected directly into the site of interest without having to be implanted.

4.1 Particles (Nano and Microparticles)

Gelatin has been widely used as a particulate formulation material, providing different characteristics suitable for the design of strategies to improve the regeneration of bone tissue.

Nanoparticles and microparticles differ in their diameter size. Polymeric nanoparticles are considered colloidal solid carriers of size between 1 and 1000 nm, while the microparticles have a diameter of several microns. In these systems, an inner core of oily or aqueous phase is covered by a polymeric membrane. When the central nucleus is composed of a vesicular system, they are called capsules (nanocapsules or microcapsules), whereas the spheres are matrix systems where the bioactive molecules are usually dispersed uniformly [95]. One of the most interesting characteristics of microparticles is their large surface area which makes them suitable for the exchange of nutrients and waste products improving the viability of enclosed cells. This property makes them useful as vehicles for cell therapy even for large bioactive molecules to be released at the site of interest [96].

In recent years different methods have been optimized to design and manufacture gelatin particles intended to use in the field of tissue engineering. The best-known methods of preparing nanoparticles are two-step desolvation, simple coacervation, solvent evaporation, microemulsion, nanoprecipitation and self-assembly technique [97]. As far as the production of gelatin microparticles is concerned, the widest method used has been to date water-in-oil-emulsion technique, followed by a crosslinking process. Nevertheless, a great deal of effort is being made to optimize more advanced production processes, to create formulations with improved surface properties [98].

In a work carried out with gelatin type A modified with succinyl groups, it was possible to synthesize nanoparticles mineralized with crystals of HA, thanks to complexation of calcium ions produced on the surface of the gelatin [29]. In fact, the composite formed by gelatin and HA has achieved satisfactory results in many studies carried out to analyze the osteoregenerator capacity of this combination [99]. As shown in Figure 3, uniformly spherical microparticles of gelatin-HA ranging in size from 5 to 10 μm showed 90% of new bone formed in bone defects caused in Sprague-Dawley rat calvaria model. In the cytotoxicity studies with

human osteoblast-like cell line G-292, the results showed minimal cytotoxic effects, considering as non-toxic for tissue engineering [100].

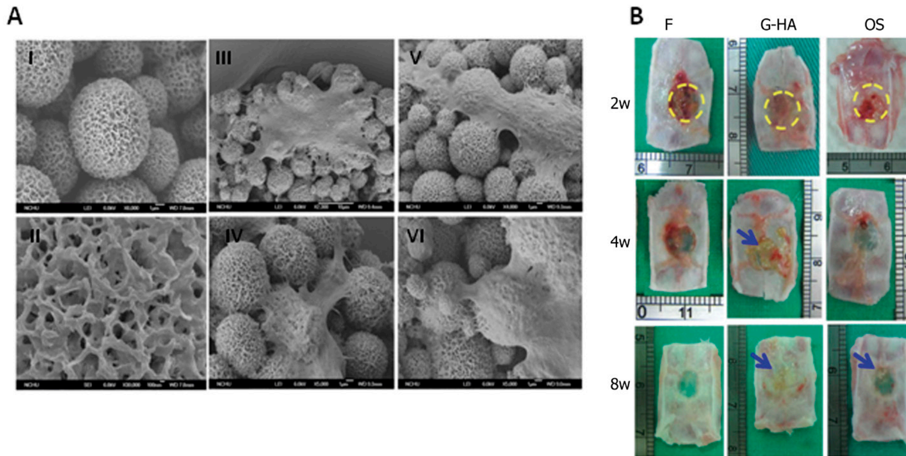


Figure 3. Gelatin-hydroxyapatite (G-HA) composite microspheres for hard tissue repair. SEM images of G-HA microspheres (AI and AII) and osteoblast-like cells on the surface of microspheres (AIII-AVI) after 14 days of culturing. (B) Photography of calvarial defects after the implantation with fibrin glue (F), G-HA and Osteoset® Bone Graft Substitute (OS) for 2 weeks, 4 weeks and 8 weeks. Adapted, with permission, from [100].

Overall, it is fairly easy to introduce particular motifs into the structure of the gelatin. This facilitates functionalizing the material to achieve selective targeting to specific tissues. Farbod K. *et al.* conjugated successfully biocompatible gelatin nanoparticles with bone-targeting alendronate to create formulations targeted to mineralized tissues. Biphosphonate groups of alendronate present strong affinity to the mineral phase of bone tissue [101].

Another desirable application of gelatin particles when designing prototypes aimed at using for bone regeneration is the combination of these 3D spherical structures with synthetic polymers, with the aim of improving the biodegradation profile of these materials. The incorporation of gelatin nanoparticles into nanofibrous scaffolds composed of polycaprolactone has been shown to result in more efficient degradation and reabsorption rates in simulated body fluid, obtaining 7 μm length nanofibers in less than 8 weeks. Furthermore, the presence of these nanoparticles increases the pore size of the scaffold and the cellular infiltration of hMSCs into the same is improved [102].

The use of gelatin microparticles in cell delivery systems turns out to be an interesting strategy to improve the effectiveness of cell therapy. Incorporation of empty gelatin microparticles into cell-containing hydrogels has been shown to serve as anchoring sites after enzymatic degradation of them, acting as porogen and cell attachment sites [103].

In recent years, some authors have focused on studying the advantages of gelatin microparticles in the osteogenic differentiation of hMSCs [103-106]. Promisingly, the incorporation of microparticles of gelatin in mesenchymal stem cell spheroids does not change the organization of the cells, although significantly impacts spheroid mechanical properties. In fact, the presence of these microparticles makes the cellular microenvironment more rigid facilitating the differentiation to lineages present in stiffer tissues, such as bone [105].

Similarly, the effects on MMP activity and cell differentiation induced by incorporating gelatin methacrylate microparticles into embryonic stem cell aggregates was investigated *in vitro*. The expression level of MMP was increased, which facilitates the remodeling of the ECM and the control of cell differentiation. These results suggest the possibility of modulating mesenchymal morphogenesis and stem cell differentiation by incorporating degradable particles based on ECM materials by modifying the activity of proteases [106]. Another strategy to control the degree of differentiation relies on the use of gelatin particles loaded with specific growth factors with the objective of creating morphogenic gradients within the pluripotent stem cell aggregates [107].

Gelatin particles have also been explored as a vehicle for therapeutic agents for tissue engineering and regenerative medicine purposes. In fact, the electrostatic properties and proteolytic degradation make gelatin quite unique to design formulations for sustained release of biologically active factors. As an example, several works have studied the potential impact of bone morphogenetic protein-2 (BMP-2) containing gelatin particles to promote and accelerate bone regeneration.

When BMP-2 is loaded into the gelatin particles and prolonged *in vitro* release thereof is achieved, the released protein remains bioactive, resulting in a significant increase in bone formation [108]. The release profile has an initial minimal burst effect followed by a linear release kinetics. There are several factors that affect the profile of this kinetics. The dose has limited effect on the release pattern whereas the isoelectric character of the gelatin has a fundamental role due to the ionic complexion that is created between them. The crosslinking degree of the particles also affects the kinetics since the controlled release is based on the enzymatic degradation of the gelatin [109]. One of the efficient strategies to achieve extended sustained release of this osteogenic protein is the possibility to combine gelatin with heparin, as it provides binding sites and stabilizes the growth factor, allowing greater protection against denaturation and proteolytic degradation [110]. Another possibility for improving control over the release is to form microparticles with gelatin modified with methacryloyl groups since potent platform for the controlled release of electrostatically bound growth factors for emerging tissue engineering approaches will be achieved [111].

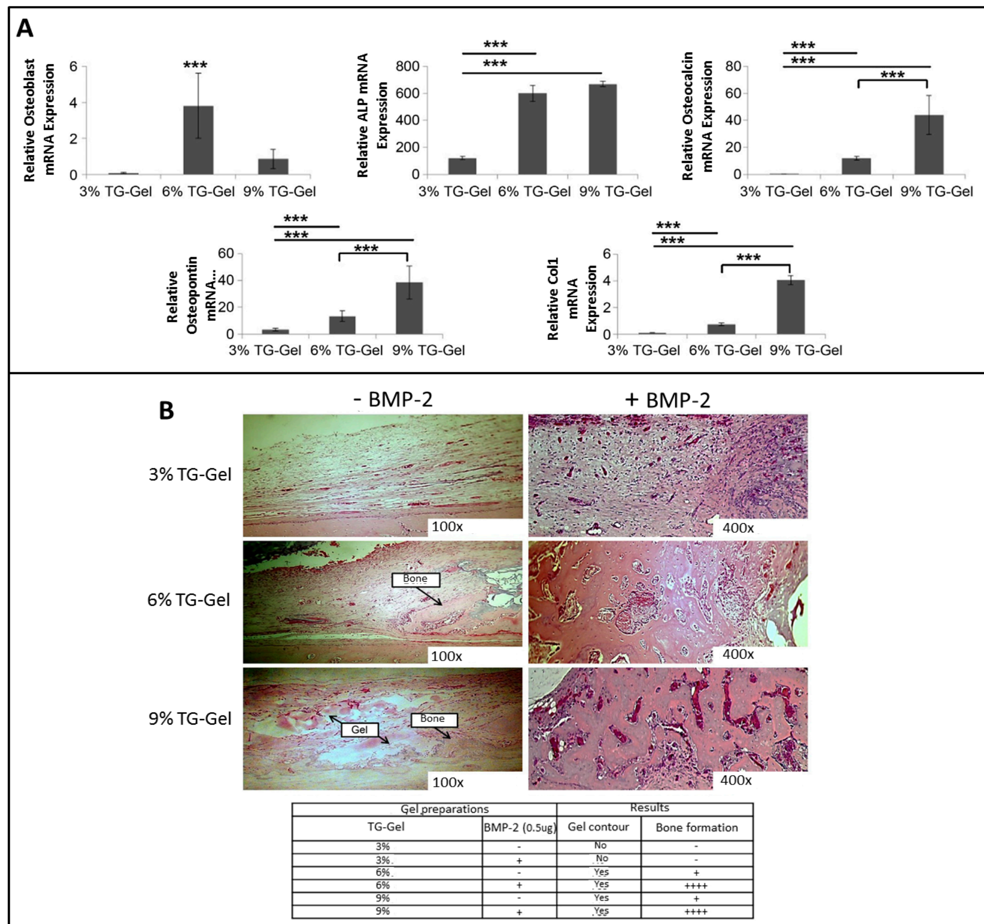
Designing multiple growth factors delivery systems could be a potentially interesting therapeutic tool for enhancing the efficacy of agents involved in the regeneration process. The incorporation of microparticles loaded with different growth factors into hydrogels is an effective approach to achieve this objective. These systems have the ability to release bioactive factors in a multimodal mode [112]. For example, in a study performed with periodontal ligament fibroblasts the incorporation of gelatin microparticles loaded with either BMP-2, insulin-like growth factor 1 (IGF-1), or a mixture of both microparticles into macroporous scaffolds resulted in greater effects of ALP activity, more calcium deposition and higher osteocalcin and osteopontin production. This scaffolds containing dual microparticles can be useful as a drug delivery vehicle to improve cells attachment, proliferation and osteoblastic differentiation of periodontal ligament fibroblasts in a synergistic manner [113]. In a similar study, it was concluded that the initial release of BMP-2 from a chitosan gel followed by the release of IGF-1 from gelatin microparticles may result in an effective strategy to achieve increased bone cell osteoblastic activity [114]. A very important aspect to take into account when designing dual synergetic systems with several growth factors is the dosing and release timing. As an example, depending on the dose ratios used for the combination of BMP-2 with basic fibroblast growth factor (bFGF), an increase in osteogenesis or block bone formation was achieved in rats with distal femoral condyle bone defect [73].

4.2 *In situ* forming gels

In situ gelling formulations are injectable substitutes that can be administered with minimally invasive surgical procedures. This property has contributed to join forces in developing efficient systems for tissue engineering, since giving the step to the clinic seems more feasible. Regarding the advantages of these formulations in comparison to preformed 3D scaffolds, the ability to treat irregular shaped osseous defects may be one of the most important characteristics, since they are injected directly into the injury sites thanks to their fluidity. The formulations must be able to replicate a spatially organized platform with features of bone tissue to use as bone filler for orthopedic and craniofacial reconstructions in regenerative medicine [115,116].

It is well established that *in situ* formation of the gel must be produced under physiological, fast and selective conditions. Regarding the methodologies developed to design this type of prototypes chemical crosslinking, electrostatic interactions, self-assembly strategies, as well as stimuli-response methods have achieved hopeful results [117]. Significant efforts are now being made to develop gelatin-based formulations having the optimal properties to be injected [118]. In this respect, an advanced "Host-Guest Macromer" approach has shown satisfactory results in the production of hydrogels with bioadhesive properties and the ability to retain and release hydrophobic agents. This macromer consists on the complexation between aromatic residues of gelatin and the free diffusing photocrosslinkable acrylated β-cyclodextrins.

UV irradiation is necessary for the initiation of the physical polymerization of the gelatin strands [119].



Gel preparations		Results	
TG-Gel	BMP-2 (0.5ug)	Gel contour	Bone formation
3%	-	No	-
3%	+	No	-
6%	-	Yes	+
6%	+	Yes	++++
9%	-	Yes	+
9%	+	Yes	++++

Figure 4. Effect of BMP-2 and matrix stiffness on osteogenesis. *In vitro* and *in vivo* osteogenesis analysis with gelatin-mTG (TG-Gel) *in situ* gelling formulations. (A) Evaluation of osteogenic differentiation of C2C12 cells encapsulated in TG-Gel in medium with or without BMP-2 supplementation by osteogenic transcript levels: osteoblast, ALP activity, osteocalcin, osteopontin and collagen 1 (*Col1*). The mRNA expression of TG-Gel in the medium supplied with BMP-2 is normalized with respect to those in the medium without BMP-2 supplementation. Data are mean ± standard deviation, statistical differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). (B) Comparison of bone formation in cranial overlay model after 28 days of subcutaneous injection of TG-Gel formulations with or without supplementation of BMP-2. -: no bone, +: 1-25% bone, ++: 25-50%, +++: 50-75%. ++++: 75-100% bone. Adapted, with permission, from [123].

Promisingly, Sandeep T. *et al.* have developed another gelatin-based hydrogel that does not require any external energy input, catalyst or polymerization initiator, while the crosslinking occurs in non-toxic conditions. In this case, the polymerization arises from the inverse electron demand Diels-Alder click reaction occurred between the functional groups tetrazine and norborene attached to the gelatin strands [120]. Importantly, the mechanical properties of these hydrogels can be tuned for even soft and stiff tissues regeneration [121].

As mentioned above, the use of enzymes is especially interesting to achieve specificity when crosslinking the gelatins strands. Injectable gelatin hydrogels crosslinked with mTG enzyme may have adequate mechanical properties to be used in bone regenerative medicine as temporary substrates. In addition, synergic effects arise with the incorporation of specific growth factors (Figure 4). Osteoconductive formulations capable of forming new bone in cranial defect sites are fabricated using this enzyme as the crosslinking agent [122,123].

Another interesting strategy that seems to be interesting to facilitate defect closure and formation of new bone, it is the addition of calcium components in the gelatin based injectable formulations [74]. For example, calcium phosphate powder can provide the hydrogel with capacity for the osteogenic differentiation of stem cells and improve the mechanical properties of the system. Recently, an advanced hydrogel-ceramic composite has been successfully designed combining fish scale-derived calcium phosphate with gelatin-3-(4-hydroxyphenyl) propionic acid and carboxymethyl cellulose-tyramine hydrogel system [124].

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This review offers an extensive overview of the potential of gelatin as a biomaterial for its use in bone tissue repair and regeneration. Regarding the possible formulations that can be prepared with gelatin as the main material, 3D porous scaffolds and nanofibrous implantable systems are more appropriate for large bone defects. On the other hand, for the reconstruction of irregular defects, it seems that the injectable materials can provide better results. These formulations are based on semi-liquid materials that are polymerized *in situ* in physiological conditions. Despite the significant progress has been made already, efforts must continue to move this biomaterial forward to routine clinical practice. Promisingly, advances in the successful development of 3D bio-printing systems can yield satisfactory results in the design of irregular prototypes based on the results obtained from the imaging techniques of the defect. Further progressions in regulatory concerns related to safety and reproducibility, as well as improvement in fabrication methods, for example by designing operator-free technologies, will help to translate novel advanced gelatin-based formulations into the clinic. Operator-free technologies are fabrication processes developed with the aim of minimizing the manipulation by the operator and thus reduce experimental errors and improve reproducibility.

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Objectives

Gelatin is a natural origin polymer derived from collagen with exceptional properties to design and develop drug delivery carriers and three-dimensional (3D) scaffolds that serve as support for drug delivery and cell-based therapies. The capacity for ionic complexation with oppositely charged growth factors and the functional motifs for cell adhesion present in its structure have prompted the exploration of this material as a candidate for several therapeutic approaches.

Despite some limitations of this biomaterial for the development of structures guided for hard tissues regeneration, the great versatility to create advanced functional composites with tailored features, combining gelatin with other materials, makes gelatin a promising candidate for bone tissue engineering approaches. In fact, taking precisely the advantage of the great adaptability of the final characteristics, this material could serve as a base component to address not only the damaged bones, but also bone adjacent transitional regions where graded compositional, structural and biological features crosswise, such as the tendon-to-bone interface.

Assuming this, the main objective of this Doctoral Thesis is the design of a gelatin-based versatile platform to develop 3D systems for bone tissue regeneration including tendon-to-bone interface tissue engineering.

To accomplish this purpose, three specific objectives are considered:

1. Development and characterization of enzymatically crosslinked gelatin 3D scaffold with capacity to promote cellular activities and to release growth factors.

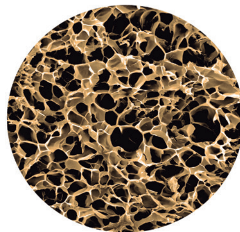
2. Development, characterization and *in vivo* assessment of osteoconductive gelatin 3D scaffolds reinforced with hydroxyapatite or calcium sulfate.

3. Development and characterization of gelatin-based composites hydrogels with anisotropic structural characteristics and integration of the dissimilar structural and compositional features of the tissue interfaces into a single gelatin-based biphasic hydrogel.

Experimental section

EXPERIMENTAL SECTION

CHAPTER 1



CHAPTER 1

Enzymatic Crosslinked Gelatin 3D Scaffolds for Bone Tissue Engineering

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ABSTRACT

Bone tissue engineering is an emerging medical field that has been developed in recent years to address pathologies with limited ability of bones to regenerate. Here we report the fabrication and characterization of microbial transglutaminase crosslinked gelatin-based scaffolds designed for serving as both cell substrate and growth factor release system. In particular, morphological, biomechanical and biological features have been analyzed. The enzyme ratio applied during the fabrication of the scaffolds affects the swelling capacity and the mechanical properties of the final structure. The developed systems are not cytotoxic according to the biocompatibility tests. The biological performance of selected formulations was studied using L-929 fibroblasts, D1 MSC and MG63 osteoblasts. Moreover, scaffolds allowed efficient osteogenic differentiation and signaling of MSC. MSC cultured on the scaffolds not only presented lower proliferative and stemness profile, but also increased expression of osteoblast-related genes (*Col1a1, Runx2, Osx*). Furthermore, the *in vitro* release kinetics of vascular endothelial growth factor (VEGF) and bone morphogenetic protein -2 (BMP-2) from the scaffolds were also investigated. The release of the growth factors produced from the scaffolds followed a first order kinetics. These results highlight that the scaffolds designed and developed in this work may be suitable candidates for bone tissue regeneration purposes.

Keywords: gelatin, scaffold, bone, tissue engineering, drug delivery.

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1. INTRODUCTION

The bone tissue has a unique property of self-remodeling and regeneration (Hayrapetyan, *et al.* 2015). However, there are situations in which the capacity for regeneration of the tissue is insufficient and unsatisfactory as a method of healing (Dimitriou, *et al.* 2011). Nowadays, the "gold standard" strategy for the treatments of these pathologies results from autologous bone grafting. Nevertheless, several complications are associated with this method including donor site morbidity, prolonged periods of hospitalization and rehabilitation and increased risk of deep infections (Jayash, *et al.* 2017). As a consequence, an immense effort has been made in order to develop novel therapies that could shed light on this challenge. The interdisciplinary field of tissue engineering and regenerative medicine aim to provide potential solution to injured or diseased tissues by developing functional substitutes that maintain, restore or enhance tissue function. This therapeutic arsenal is based mainly on the use of recombinant proteins, cells, and biomaterials (Guan, *et al*. 2017).

In the case of bone regeneration, development of porous three-dimensional (3D) structures that act as scaffolds to carry and release those therapeutically active agents could be an interesting strategy. The main purpose of the scaffolds will be to replace temporally the function of the biological tissue and promote the formation of the new bone (Roseti, *et al.* 2017). In this context, some researchers have proposed the combined use of angiogenic and osteogenic factors as a strategy for the promotion of bone healing. The success for the regeneration of bone tissue using vascular endothelial growth factor (VEGF) and bone morphogenetic protein -2 (BMP-2) together depends not only on the appropriate selection of the dose and the release kinetic, but also on the duration of the experimental study proposed in animals (Dou, *et al*. 2019; Patel, *et al*. 2008; Subbiah, *et al.* 2015).

Although many different materials have been investigated for their potential to the fabrication of these structures (Gibbs, *et al*. 2016), polymers from natural origin have been of particular interest as candidates in the development of scaffolds for bone tissue engineering (Kuttappan, *et al*. 2016). Gelatin is a natural hydrosoluble polymer that is obtained from the hydrolytic process of collagen. Gelatin shows interesting physicochemical and biological properties, make it suitable for its use in biomedicine (Echave, *et al*. 2017). One very useful property of this biomaterial is the possibility of obtaining gelatins with different isoelectric points. Both positively and negatively charged factors can form ionic complexes with gelatin strands (Yamamoto, *et al.* 2001). In addition, all types of gelatin have shown to be biocompatible (low immunogenic capacity), biodegradable and non-cytotoxic. Moreover, biologically functional amino acid sequences such as arginine-glycine-aspartic acid (RGD) motif are found in its structure, which improves cell adhesion, differentiation, and proliferation (Echave, MC *et al*. 2017). Most importantly, the ability of gelatin to form a thermally reversible

network in water can provide crosslinked network structure with sufficient mechanical and thermal stability under physiological environment (Gil, *et al*. 2005).

Recently, we reported biologically active and biomimetic dual gelatin scaffolds crosslinked with genipin (Sanchez, *et al*. 2017). However, the use of genipin as a crosslinker agent has several limitations including long crosslinking times, structural coloration and unspecific reactivity (Butler, *et al*. 2003). Scientists are scrambling to develop methods that can quickly, easily and accurately crosslink gelatin strands. One exciting alternative may be the use of specific enzymes that catalyze the covalent union of amino acids present in the material structure (Teixeira, *et al*. 2012). The enzyme microbial transglutaminase (mTG) catalyzes the production of an isopeptide bond between a glutamine and lysine residues, without the need for calcium as a cofactor (Chen, *et al*. 2014). Previous studies have reported the use of this enzyme to obtain covalent crosslinking within stable gelatin hydrogels (M. Hu, *et al*. 2017; Yang, *et al*. 2018).

The primary aim of this study is the design, development and characterization of gelatin scaffolds crosslinked with mTG. Morphology and biomechanical properties have been addressed as well as the biological characteristics of the scaffolds on different cell types. Furthermore, the capacity of retention and release of key growth factors in bone remodeling, such as VEGF and BMP-2 have also been examined *in vitro.*

2. MATERIALS AND METHODS

2.1 Materials

Gelatin from bovine skin Type B with ≈ 225 bloom strength, Cell Counting Kit-8 (CCK-8) and Collagenase P were purchased from Sigma-Aldrich (Spain). mTG derived from *Streptoverticillium mobaraense* with a specific activity of 100 U/g was kindly supplied by Ajinomoto Foods Europe (France). Mouse L-929 fibroblasts and mouse D1 ORL UVA Mesenchymal Stem Cells (MSCs) cell lines, Dulbecco's Modified Eagle's Medium (DMEM 30-2002), horse serum and Eagle's Minimum Essential Medium (EMEM 30-2003) culture mediums were obtained from ATCC (Spain). MG63 cells were immortalized osteoblasts isolated from a 14 years-old caucasian male osteosarcoma. Trypsin, Hoechst 33342, foetal bovine serum (FBS), phosphate buffered saline (PBS) pH=7.4 (1X) and penicillin-streptomycin were purchased from Fisher Scientific (Spain). rhVEGF was kindly supplied by Agrenvec (Spain). Finally, VEGF ELISA kit, BMP-2 ELISA kit and rhBMP-2 were obtained from Peprotech (UK).

2.2 Cell Culture

The cells used to test the biological performance of the formulations were cultivated with a specific culture medium. Mouse L-929 fibroblasts were cultured in EMEM 30-2003 growth

medium supplemented with horse serum 10% (v/v) and 1% (v/v) penicillin-streptomycin. For the culture of D1 MSCs, the DMEM 30-2002 medium was supplemented with FBS 10% (v/v) and 1% (v/v) penicillin-streptomycin. MG63 osteoblasts were cultured with DMEM culture medium supplemented with FBS 5% (v/v), L-Glutamine 1% (v/v) and gentamicin 0.5% (v/v). Primary murine MSC from bone marrow (BM-MSCs) were used to analyze the influence of the scaffolds on the progression in osteogenic differentiation of these cells. The isolation of the cells was achieved from the femurs of 6-8 week old mice, as previously described elsewhere (Aquino-Martinez, *et al.* 2016; Soleimani and Nadri 2009). Briefly, soft tissues were cleaned and the femurs kept in complete media (DMEM supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine). The femur ends were cut and the bone marrow was flushed and collected. Cell suspension was filtered with a 70 μm cell strainer (Falcon, USA), transferred to a 100 mm cell culture plate and incubated at 37 °C. The media was changed after 24 hours and then every eight hours for two to three days to discard non-adherent cells. After five to seven days, when the adherent cells reached 75%-80% of confluence, the cells were washed three times with warmed PBS and trypsinised for three minutes at room temperature. The lifted cells were cultured and expanded in complete media. For osteogenic differentiation, cells were cultured in α-MEM containing FBS 10% (v/v), penicillin-streptomycin 1% (v/v), L-glutamine 2 mM, sodium pyruvate 1 mM with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid for 10 days. All cell cultures were kept at 37 °C in a humidified atmosphere of 95% air with 5% CO_2. The split of the cells was performed routinely when cells reached confluence.

2.3 Development of enzymatically crosslinked gelatin-based 3D scaffolds

Gelatin-based 3D scaffolds were prepared by the enzymatic crosslinking of gelatin with mTG. Briefly, a homogeneous dispersion of gelatin was prepared by dissolving Type B gelatin powder in distilled water at 40 °C under constant stirring for 45 minutes. Simultaneously, the mTG solution was prepared in deionized water at room temperature (100 mg/mL). Then, the required volume of the enzyme solution was added to the gelatin solution and mixed for another 5 minutes. Nine different formulations were prepared varying the final concentration of gelatin (10, 15 and 20 %w/v) and the enzymatic activity (10, 20, 30 U/g gelatin) (Figure 1A). Subsequently, hydrogel solution (5 mL) was casted into 100 mm Petri dishes and the formulations were kept at 4 °C for 1 hour in order to get fully crosslinked hydrogels. Afterward, the hydrogels were punched out to obtain 8 mm diameter 3D scaffolds. These scaffolds were immersed into ethanol 70% (v/v) for 10 minutes and thereafter they were washed twice in PBS. Finally, the scaffolds were frozen at -80 °C and freeze-dried for 42 hours.

2.4 Swelling behavior and Mechanical properties

A swelling study was carried out to determine the ability of the scaffolds to absorb and retain water. The swelling ratio was calculated using equation 1; where Ws and Wo are the weight of wet and freeze-dried scaffolds, respectively. First, dry scaffolds were weight prior to immersion into PBS solution. They were maintained at 37 ˚C and under constant shaking (300 rpm) for 24 hours. Afterward, the excess surface water of the wet scaffolds was gently removed with a filter paper and then the scaffolds were weighed again. Four different samples were used for each type of scaffold, and the average value was used for further analysis.

Equation 1: Swelling ratio = (Ws – Wo)/Wo

The mechanical properties of enzymatically crosslinked scaffolds were defined with Instron Microtester 5548 equipment with a precision of 0.001 mm and 0.0001 N in displacement and force, respectively. Young´s modulus was determined by uniaxial unconfined static compression test using eight replicates previously rehydrated with PBS. A protocol described by Acosta Santamaría (Acosta Santamaría, *et al.* 2013) was followed to complete the mechanical properties characterization.

2.5 Scanning Electron Microscopy (SEM)

The morphology and microporous structure of the fabricated 3D scaffolds were assessed using SEM. Images of the surface and transverse section of the scaffolds were acquired to assess the structure of the formulations and see the arrangement of the pores throughout them. The dried scaffolds were covered with carbon adhesive tape and they were examined by Hitachi S4800 microscope.

2.6 Biocompatibility study

The cytocompatibility of the scaffolds was evaluated by using direct and indirect tests, following the guideline ISO 10993 (Biological evaluation of medical devices guideline: cytotoxicity on extracts and cytotoxicity by direct contact). These assays were performed using L-929 fibroblasts. To evaluate the toxicity produced by cell-scaffold direct interaction, 3.5×10^4 cells were seeded in a 24 wells plate for 24 hours. Concurrently, dry scaffolds were immersed in 1 mL of complete culture medium for 24 hours, in order to get hydrated samples. Thereafter, direct contact between cells and scaffolds was achieved by placing a hydrated scaffold above each cell-containing wells and they were cultured for 24 hours more. On the other hand, 5×10^3 fibroblast cells per well were plated in a 96-well cell culture plate to evaluate indirect cytotoxicity of the scaffolds. Simultaneously, freeze-dried scaffolds were immersed into growth medium for 24 hours under continuous shaking in order to get the extracts. After the incubation, cell culture medium of fibroblast cells was replaced by scaffold´s extracts and maintain for 24 hours. In both experiments metabolic activity of the cells was evaluated

using the CCK-8 assay, following kit guideline. This assay is based on sensitive colorimetric to determine the number of viable cells in cell proliferation and cytotoxicity assays. The reagent is decreased by mitochondrial dehydrogenases in cells to form an orange-colored product (formazan). The amount of the formazan dye generated by the activity of mitochondrial dehydrogenases in cells is directly related to the number of viable cells in culture. For that, absorbance was measured by a microplate reader Tecan Infinite M2000 at 450 nm. The mean absorbance value obtained in the control wells was considered as 100% cell viability. Cells seeded in control wells were not in contact either with scaffolds directly or with the extracts.

2.7 Biological performance of 3D scaffolds

The biological performance of the developed formulations was assessed concurrently with both established cell lines and primary cells. In the cell lines case, L-929 fibroblasts, D1 MSCs and MG63 osteoblastic human origin cells that have been widely used in bone tissue engineering purposes were tested. These cells were seeded on the surface of the scaffolds and at different time intervals (4, 24 and 72 hours) the cellular metabolic activity determination and nuclei staining was performed in order to analyze the cell density in each sample. Briefly, the scaffolds were exposed to UV light for 10 minutes and $20x10^3$ cells/scaffold were seeded. The surface of the polystyrene plate was taken as 2D control. For the analysis, the culture medium was removed to discard the non-adhered cells and metabolic activity of the remaining cells was analyzed by the CCK-8 assay following the manufacturer's indications. Besides, cells were fixed in formaldehyde for 10 minutes at room temperature and Hoechst reagent was added at a 10 mg/mL concentration. Finally, the nuclei were observed using an inverted fluorescence microscope (Nikon TMS). Nuclei counting was carried out using 4x objective and 4 images per scaffold were analyzed using Image J software. In addition, primary BM-MSCs from mice were used to analyze the influence of the scaffolds on the progression in osteogenic differentiation of these cells.

2.8 Western blot

Cell lysates from BM-MSCs were resolved on PAGE and transferred to Immobilon-P membranes (Millipore). Primary antibodies toward phosphorylated ERK (Cell Signalling 9102), phosphorylated p38 (Cell Signaling, 9211), phosphorylated S6 (Cell Signaling, 2211) and β-actin (Abcam ab6276) were used at a 1:1000 dilution. Immuno-reactive bands were visualized using horseradish-peroxidase-conjugated secondary antibodies and an EZ-ECL kit (Biological Industries, Cromwell, CT, USA).

2.9 Gene expression analysis

Total RNA was extracted from BM-MSCs by TRIsure reagent (Bioline). Two µg of purified RNA was reverse-transcribed by the use of a High-Capacity cDNA Reverse Transcription

Kit (Applied Biosystems). Quantitative PCRs were performed on ABI Prism 7900 HT Fast Real-Time PCR System with SensiFAST Probe Hi-ROX Mix (Bioline) and TaqMan 5´-nuclease probe method (Applied Biosystems) to measure *Nanog, Oct4* (Pou5f1), *Osx* (Sp7), *Runx2* and *Col1a1* mRNA expression. All transcripts results were normalized to TATA binding protein (*Tbp*) expression.

2.10 *In vitro* Release of Growth Factors

The release of growth factors assay *in vitro* test was completed with two relevant molecules that have demonstrated efficacy in generating substitutes for bone regeneration: VEGF (K. Hu and Olsen 2017) and BMP-2 (El Bialy, *et al.* 2017).

First, 300 ng of VEGF and 1000 ng of BMP-2 were incorporated into the formulations, adding the required volume over the dried scaffolds. They were left incubating at 37 ˚C for 12 hours, for the adsorption process. Thereafter, scaffolds were washed with 1 mL PBS under constant shaking in order to remove unbound protein fraction. The encapsulation efficiency (EE) was calculated following the equation 2, where D is the amount of the protein added and unloaded protein is the amount of the protein detected in the washing sample. Then, 1 mL of PBS was added to the tubes containing scaffolds and the sampling phase was started, taking the samples at different time intervals. The specimens were frozen at -80 ˚C, prior to measuring the growth factor concentration. To conclude the study, a solution of 0.5 mg/mL collagenase P was added to the tubes in order to degrade the scaffolds and quantify the remaining amount of the protein without releasing.

Equation 2: $EE= ((D - \text{unloaded protein}) / D) * 100$

During this assay, Protein LoBind Eppendorf tubes were utilized and there were kept at 37 ˚C under orbital shaking at 40 rpm. The quantification of growth factors contents in the samples was performed with commercially available Quantikine colorimetric sandwich ELISA kits following the instructions of the manufacturer.

The experimental data obtained from the *in vitro* release assays were fit to the first-order kinetic mathematical model (equation 3 and 4) using the non-linear regression module of GraphPad Prism software, (version 7.0). $\%\ VEGF_{released}$ and $\%\ BMP\text{-}2_{released}$ values correspond to the release of the cumulative percentage of growth factor at time *t*. K is the first-order rate constant expressed in $hour^{-1}$ and $\%\ VEGF_{released\ \infty}$ and $\%\ BMP\text{-}2_{released\ \infty}$ correspond to the maximal percentage of the growth factors released at maximum interval.

Equation 3: $\%\ VEGF_{released} = \%\ VEGF_{released\ \infty}\ (1 - exp^{-K \cdot t})$

Equation 4: $\%\ BMP\text{-}2_{released} = \%\ BMP\text{-}2_{released\ \infty}\ (1 - exp^{-K \cdot t})$

2.11 Data analysis and statistics

Data are presented as mean ± SD (standard deviation) shown by the error bars. Statistical analysis was performed by GraphPad Prism software, version 7.0. Shapiro-Wilk test was used for Normal distribution determination. Statistical analysis was carried out by one-way Analysis of Variance (ANOVA) followed by the Tukey *post hoc* test for multiple comparisons in normally distributed data. Student's *t*-test was employed for differences determination between two groups. In case the data did not fit the normal distribution, the nonparametric Kruskal-Wallis one-way analysis of variance was utilized. Statistical significance between groups was considered at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Swelling Behavior and Mechanical Properties

Nine different enzymatically crosslinked gelatin-based 3D scaffolds were successfully fabricated by freeze-drying technique. Gelatin concentrations extended from 10 to 20 % w/v whereas crosslinking proportions ranged between 10 and 30 U/g gelatin. The mTG enzyme catalyzes the isopeptide bonds between the γ-carboxamide groups of glutamyl residue and the ε-amino groups of the lysine residue (Figure 1A). Its catalytic activity is independent for the presence of calcium ions, being able to achieve the stable covalent crosslinking of the gelatin fibers in soft conditions during the fabrication of gelatin-based hydrogels for regenerative purposes (Alarake, *et al*. 2017).

Both dried and rehydrated scaffolds in PBS were weighted in order to calculate the swelling ratio following equation 1. All the formulations proved to have a great capacity of hydratation during the assay, going from whitish opaque structures to completely transparent systems (Figure 1B). The swelling ratio was significantly lower in cases in which the enzyme ratio was higher (Figure 1C). The scaffold with the greatest swelling capacity was composed of 10 % gelatin and 10 U/g of mTG showing the ratio of 12.19. On the other hand, the prototype with the highest concentration of gelatin and enzyme showed the lowest ability to absorb water with the swelling ratio of 6.11. In fact, the increase of polymer concentration hinders the water penetration into the hydrogel structure. Consequently, our results are in accordance with previous works that have confirmed that more enzymatic activity of mTG turns out to present less ability to retain water (Alarake, *et al*. 2017).

Inversely, the stiffness of the structures was significantly higher when the concentration of the gelatin and the level of hydrogel crosslinking was increased (Figure 1D). These results are in accordance with previous work, where the crosslinking grade sets the elasticity of the developed matrix (Engler, *et al*. 2006). Therefore, the formulations containing 20 % of gelatin

and 20 U/g and 30 U/g mTG presented Young Modulus values of 38.7 ± 2.2 and 41.9 ± 8.0 respectively. The elasticity values of the developed hydrogels are on a par with osteoid tissue mechanical characteristics (Zaky, *et al*. 2017). In fact, this tissue is a non-mineralized transient structure which is produced first during bone tissue formation prior to been replaced by the mineralized secondary bone (Lopes, *et al.* 2018).

3.2 Biocompatibility Study

In the biocompatibility studies, 100% of the viability was considered the absorbance value obtained in the CCK-8 assay with the control samples that were not in direct contact with the scaffolds or the extracts.

In the cytotoxicity assay on extracts, all scaffolds achieved cell viability greater than 100%. In contrast, all the scaffolds showed lower levels of viability in the toxicity study by direct contact between cells and scaffolds (Figure 1E). This effect may be due to the presence of RGD sequences in the surface of the scaffolds, since these integrin-binding domains are present in the innate structure of the gelatin (Yue, *et al*. 2015). Focal adhesions between the cells and the gelatin fibers are produced by the binding of integrins present in the cellular cytoplasmic membrane with those sequences mentioned above (LeBaron and Athanasiou 2000). Such an effect in gelatin-based structures has been previously described. Even so, in all cases the L929 fibroblasts viability was above 70%, the limit required by ISO guidelines to consider as non-cytotoxic for *in vitro* tests. Besides, the increase of the enzyme activity during the preparation of the systems does not come from a significant decrease in cell viability.

3.3 Morphological Analysis

Based on the results obtained in the swelling, stiffness determination and biocompatibility studies, two prototypes were selected for further *in vitro* characterization: 10% gelatin + 20 U/g (GEL_10/20) and 20% gelatin + 20 U/g (GEL_20/20). Even though the cytocompatibility values for both formulations were similar, significant differences were determined in relation to their stiffness and ability to swell. Indeed, GEL_10/20 formulation presented higher capability to absorb water while GEL_20/20 was twice stiffer.

The images obtained by SEM revealed random conformation of gelatin fiber densely packed with the presence of pores distributed throughout all surface that allowed the rehydration. In this study, 3D scaffolds with different concentrations of gelatin and the enzyme mTG have been fabricated by freeze-drying technique. This fabrication method is a useful procedure to create pores in the structure (Garg and Goyal 2014). In fact, in the course of the preparation process, ice crystals are formed in the scaffold during the freezing phase allowing the creation of pores by the sublimation and desorption (Mano, *et al*. 2007). Descriptive images of the surface and the cross-section of the formulations are represented in Figure 1F. In fact,

a sponge-like isotropic structure with deep pores that crossed the transversal section was obtained. In both cases, the pore size was measured in the range of 150 - 230 μm and no differences in the distribution of the pores was revealed in the microstructural analysis.

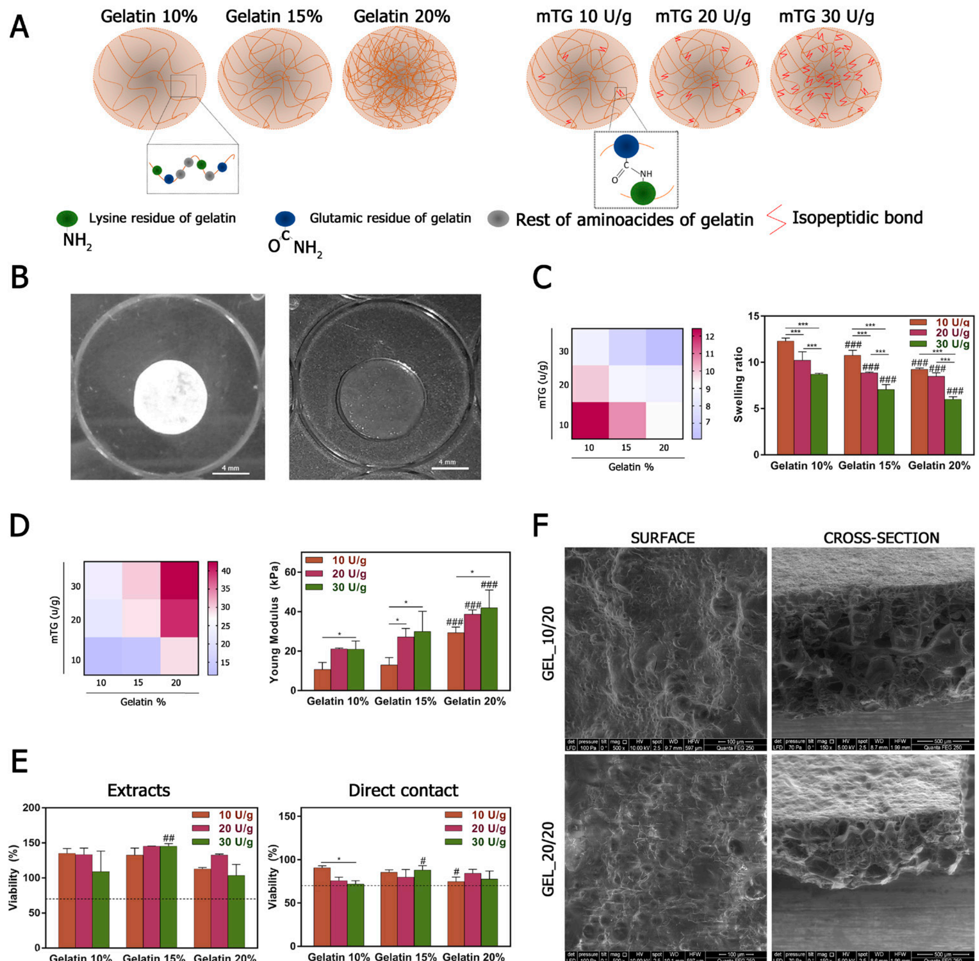


Figure 1. Characterization of enzymatically crosslinked gelatin-based 3D scaffolds. A) Schematic image of the developed formulations. Gelatin fibers are represented as amino acids chains and the covalent isopeptidic bond formed during the enzymatic crosslinking process is also depicted. B) Digital photography of both lyophilized and rehydrated 3D scaffolds. C) Swelling behavior of the systems displayed on combinatorial heat-diagram. D) Young moduli of the formulations for mechanical analysis. E) Cellular viabilities on direct contact and extracts cytotoxicity tests. F) Representative SEM images of the surface and cross-section of freeze-dried scaffolds. Statistical significance: * p<0.05, ** p<0.01, *** p<0.001 compared to the groups with the same concentration of gelatin and # p<0.05, ## p<0.01, ### p<0.001 compared to the group with the same enzymatic activity and 10% of gelatin concentration.

3.4 Culture of Cells onto 3D Scaffolds

Cell-scaffold interactions were evaluated using L-929 fibroblasts, D1 MSCs and MG63 osteoblasts. In the case of fibroblasts, the number of cells adhered at 4 hours in the three conditions was similar, without significant differences in metabolic activity. At 24 and 72 hours, the number and the activity of the cells in the control and the GEL_10/20 scaffold was similar, with a slight decrease in the case of GEL_20/20 (Figure 2A-B). Regarding the interaction between scaffolds and D1 MSCs no differences were found between the control and the two type scaffolds at 4 and 24 hours. However, a slight decrease in the case of GEL_20/20 was observed in the value obtained in the CCK-8 assay at 72 hours (Figure 2D). The distribution of the cell nuclei on the scaffolds remained homogeneous throughout the entire surface (Figure 2C). Finally, in the case of the study with MG63 osteoblasts, differences between the control and the two types of scaffolds were observed from 4 hours post-seeding. The adhesion of osteoblast was lower in both cases compared with the control, as could be seen in the images of the nuclei stains (Figure 2E). The values of the optical densities obtained in the CCK-8 assay (Figure 2F) corroborate these qualitative results.

Since both the metabolic activity and the number of adhered cells to the scaffolds have been increased during the incubation period for all the cell lines studied, it can be concluded that the developed 3D polymeric scaffolds are suitable structures to support cell growth on them.

3.5 Culture of BM-MSCs in the scaffolds increases the expression of osteogenic genes

We also examined the ability of these scaffolds to either stimulate osteogenesis or maintain the undifferentiated status of the BM-MSCs. Therefore, we cultured the cells in the scaffolds for 10 days. The gene expression quantification of the stemness markers *Oct4* and *Nanog* demonstrate that, after 10 days culture in the GEL 10/10 and GEL 20/20 scaffolds, the expression of *Oct4* and *Nanog* was reduced (Figure 3A). A parallel analysis of osteoblastic gene expression showed that the *Col1a1*, *Runx2* and *Osx* mRNA levels were significantly increased after 10 days (Figure 3A). The addition of BMP-2 during the differentiation induced a further increase in the expression of these markers, more evident for cells cultured in the GEL_10/20 scaffold.

To determine the mechanisms of BM-MSCs differentiation in the scaffolds, we analyzed intracellular signaling triggered by the cell culture in the different scaffolds. Analysis was performed after 10 days of differentiation of BM-MSCs in the scaffolds. The levels of phosphorylated ERK1/2 were decreased after 10 days suggesting a lower proliferative rate of the cells at these time points. In return, S6-kinase and p38 signaling pathways were activated after 10 days. Since activation of p38 and S6-kinase are required for osteogenic differentiation,

these results suggest increased osteogenic differentiation ability of BM-MSCs when cultured in the GEL_10/20 and GEL_20/20 scaffolds (Figure 3B). Therefore, BM-MSCs, cultured in the GEL_10/10 and GEL_20/20 scaffolds, have the ability to reduce their stemness and proliferation and promote their specification into an osteogenic program.

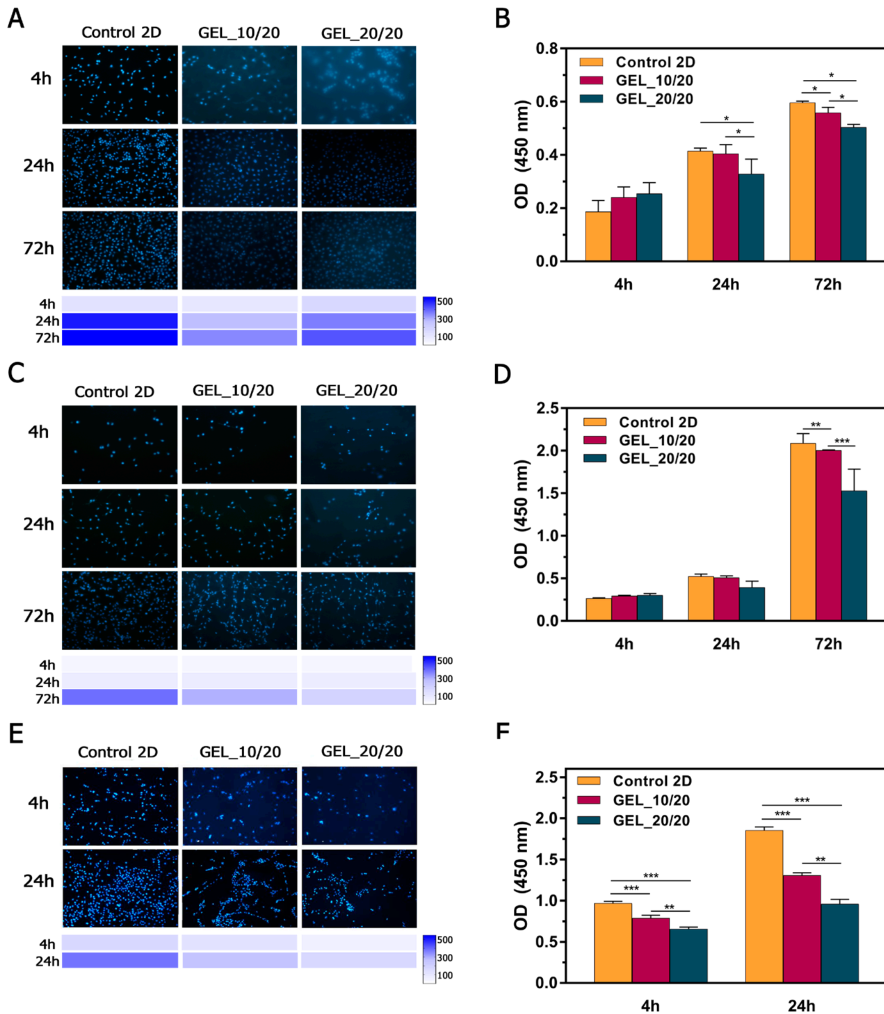


Figure 2. Biological performance of scaffolds with stablished cell lines. The mitochondrial activity of the cells seeded onto the scaffolds was determined from CCK-8 assay. The staining of the nuclei was performed at the same time intervals and the quantification from the fluorescence imaging were afterward plotted in heat-diagrams. A and B correspond to the experiments with L-929 fibroblasts. C and D present the data obtained in studies with D1 MSCs. Finally, E and F show the results corresponding to studies with MG63 osteoblasts. Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

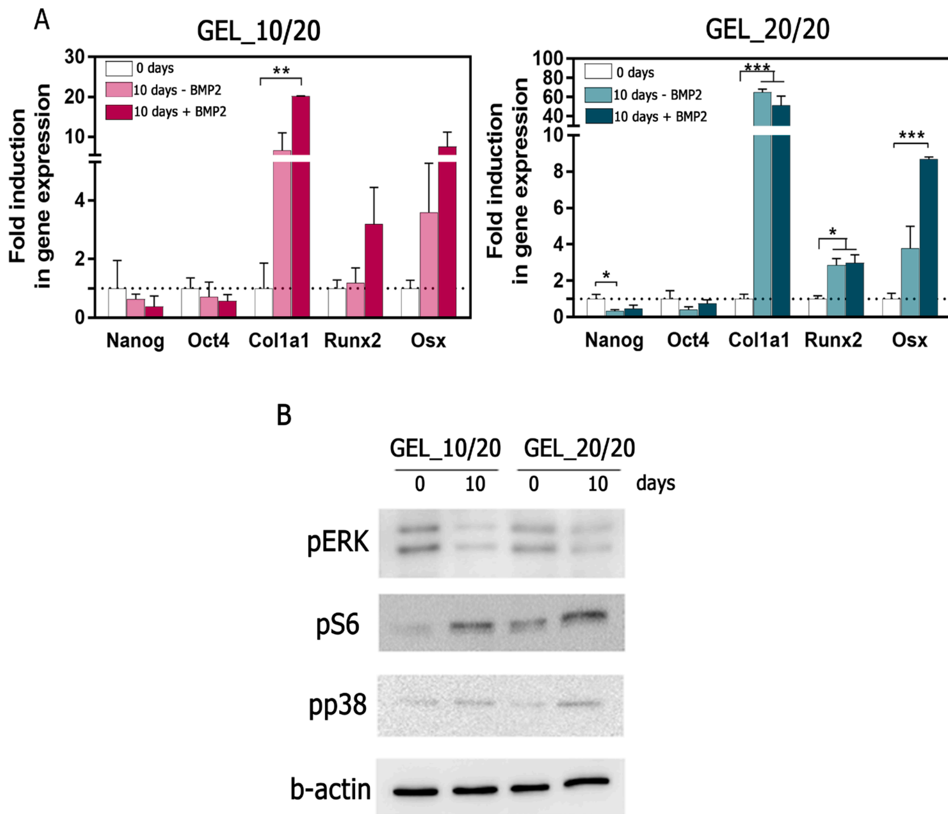


Figure 3. Scaffolds increase osteogenic marker expression. (A) Primary BM-MSCs were cultured on GEL_10/20 or GEL_20/20 scaffolds with or without 2 nM of BMP-2 for 10 days. The mRNA expression of *Nanog*, *Oct4*, *Col1a1*, *Runx2* and *Osx* was analysed and normalised to *Tbp* levels. (B) Western blot analysis of the phosphorylation levels of ERK, S6 and p38. Cells were cultured in the scaffolds for 10 days. Data was quantified relative to the levels of b-actin. Differences were considered significant at *p* values: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ when compared to control cells at time 0.

3.6 *In vitro* Release of Growth Factors

An angiogenic growth factor, VEGF, and BMP-2 as a potent osteoinductive factor were chosen for the *in vitro* release studies from GEL_10/20 and GEL_20/20 scaffolds. Successful bone healing relies on several biological processes that act in a controlled spatiotemporal manner. These include inflammation, soft callus formation, cartilage turnover and bone remodeling (Marsell and Einhorn 2011). The VEGF protein is present in almost all the phases of bone repair (K. Hu and Olsen 2017). In fact, VEGF increases the permeability of sinusoidal endothelial cells and induces neutrophil migration during the inflammation phase (Ancelin, *et al.* 2004). This growth factor has been shown to be effective in the process of

differentiation of periosteal progenitor cells to osteoblasts (K. Hu and Olsen 2016). Meanwhile, the BMP-2 is a potent inducer of bone and cartilage formation. It has been reported that BMPs can activate pathways that stimulate VEGF synthesis (Ai-Aql, *et al.* 2008), demonstrating that there is a close relationship between both growth factors during the healing process of the bone. BMP-2 is currently the most commonly used cytokine as bone graft substitutes (McKay, *et al.* 2007) and rhBMP-2 containing lyophilized product is on the market as an alternative treatment for bone grafting for several clinical conditions (El Bialy, *et al.* 2017). Therefore, it seems an interesting strategy to use these growth factors synergistically to develop treatments for bone regeneration (Aksel and Huang 2017; An, *et al.* 2017). However, the spatiotemporal control over growth factor release, which is necessary to achieve physiological doses in the injured tissue (Anitua, *et al.* 2008), remains a considerable challenge by now.

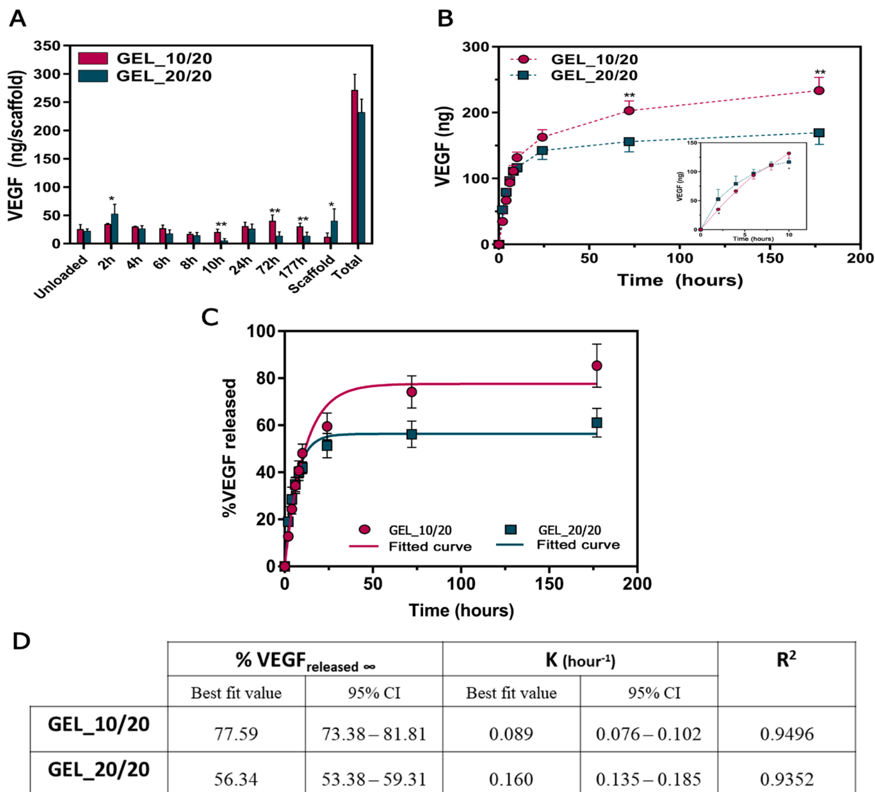
In the case of VEGF, both scaffolds showed high EE, 91.4% and 92.5% for GEL_10/20 and GEL_20/20, respectively. In the case of the GEL_10/20 scaffold, an 85.4% of the absorbed protein (233.6 ng) was released during the test period. A burst effect of 59.5% during the first 24 hours was observed in the release curve of GEL_10/20 matrices. Then, during the following days of the study, a total of 70.7 ng of VEGF released from the scaffolds were detected. In contrast, the GEL_20/20 scaffold released only 61.1% of total encapsulated VEGF (169.7 ng) until the end of the assay, being the burst effect in the first 24 hours similar (51.4%) (Figure 4A-B).

The release profiles of both groups have been fitted to the first-order mathematical model following the equation 3. As can be seen in Figure 4C the correlation for the model is appropriate, being the regression coefficients (R^2) 0.9496 and 0.9352 for the systems GEL_10/20 and GEL_20/20, respectively. Fitting parameters can be found in the table of Figure 4D.

Regarding the release of BMP-2, both types of scaffolds presented similar EE over 89%. GEL_10/20 released 614.6 ng of BMP-2 during all the kinetic period, while the prototype GEL_20/20 accumulated 443.7 ng released (Figure 5B). In the case of GEL_10/20 an initial burst effect of 56.5% was observed during the first 24 hours. After that, additional 112.0 ng of BMP-2 were released from the scaffolds until the end of the assay. Otherwise, GEL_20/20 scaffold presented an initial burst effect of 43.2% during the first 24 hours and then, a total of 57.5 ng of the growth factor was detected over the next days (Figure 5A-B). The adjustment of the BMP-2 release kinetic following the equation 4 revealed a good correlation with the model, presenting values of 0.9610 and 0.9723 for R^2 (Figure 5C). The kinetic parameters are summarized in Figure 5D.

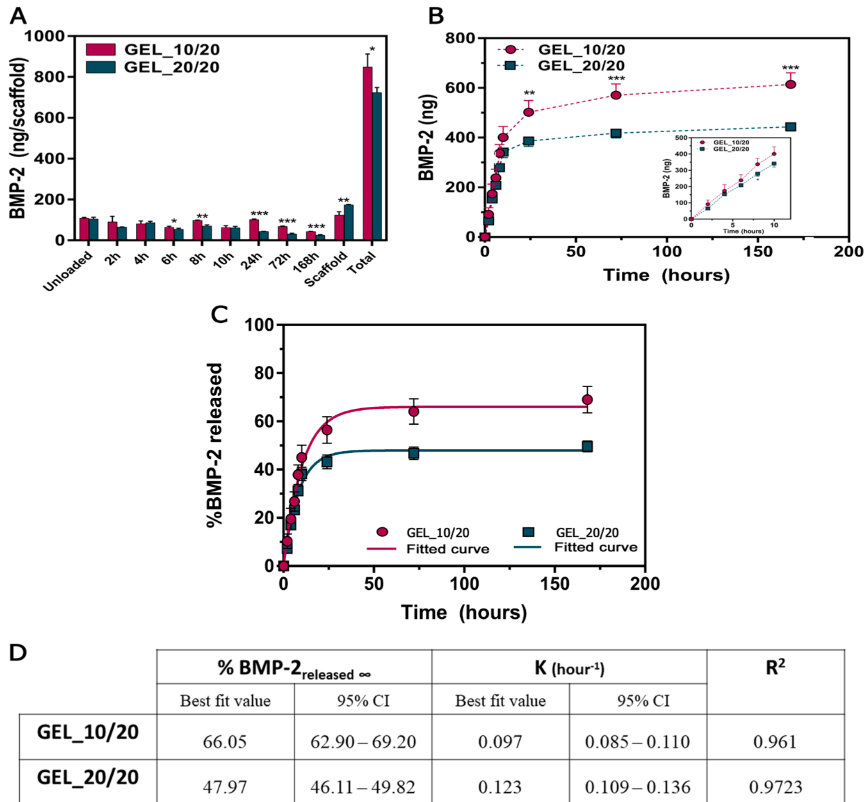
In both growth factors kinetics, the significant differences in the release of the therapeutic agents are accentuated in the second phase of the liberation process, after the burst effect. In fact, the formulation GEL_20/20 presents greater capacity for retention of the factor.

The total amounts released from these formulations are lower. The electrostatic bonding between gelatin and a wide range of bioactive molecules by the union of opposite charges has been largely used as a strategy to use this biomaterial as a drug delivery carrier. Different types of systems such as nanoparticles, microparticles or 3D scaffolds have been developed so far (Foox and Zilberman 2015). The gelatin used for the production of 3D systems in this work has an isoelectric point of 4.7-5.2. On the other hand, the isoelectric point of both BMP-2 and VEGF is higher (8.5 and 7.6 respectively). Thus, an ionic complexation between the matrix and the factors is expected to arise at physiologic pH. Since the GEL_20/20 is prepared with more amount of gelatin, more interactions between negatively charged aminoacids and these growth factors are expected.



	% VEGF$_{released\ \infty}$		K (hour^{-1})		R^2
	Best fit value	95% CI	Best fit value	95% CI	
GEL_10/20	77.59	73.38 – 81.81	0.089	0.076 – 0.102	0.9496
GEL_20/20	56.34	53.38 – 59.31	0.160	0.135 – 0.185	0.9352

Figure 4. *In vitro* release of Vascular Endothelial Growth Factor (VEGF). A) VEGF amount released at different time points, protein amount detected into the scaffolds at the end of the assay and the total protein detected during the study. B) The cumulative release of VEGF from the two prototype scaffolds analyzed. C) Adjustment of the VEGF release curve according to the first-order release kinetics equation 3 of the GEL_10/20 scaffold and the GEL_20/20 scaffold. D) Regressed parameters for the first-order kinetic model in VEGF release assay. Statistical significance between groups: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. K – First-order rate constant, CI – Confidence interval, R^2 – Regression coefficient.



	% BMP-2$_{released\ \infty}$		K (hour^{-1})		R^2
	Best fit value	95% CI	Best fit value	95% CI	
GEL_10/20	66.05	62.90 – 69.20	0.097	0.085 – 0.110	0.961
GEL_20/20	47.97	46.11 – 49.82	0.123	0.109 – 0.136	0.9723

Figure 5. *In vitro* release of Bone Morphogenetic Protein - 2 (BMP-2). A) BMP-2 amount released at different time points, protein amount detected into the scaffolds at the end of the assay and the total protein detected during the study. B) The cumulative release of BMP-2 from the two prototype scaffolds analyzed. C) Adjustment of the BMP-2 release curve according to the first-order release kinetics equation 4 of the GEL_10/20 scaffold and the GEL_20/20 scaffold. D) Fitting parameters for the first-order kinetic model in BMP-2 release assay. Statistical significance between groups: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. K – First-order rate constant, CI – Confidence interval, R^2 – Regression coefficient.

In this system, the release of the factors from the 3D gelatin scaffolds follows the first-order kinetics. The first-order mathematical model can be used to describe the dissolution of drugs in pharmaceutical dosage forms such as porous matrices containing water-soluble drugs (Dash, *et al.* 2010). In this kinetic model the release is proportional to the quantity of drug remaining in the dosage form. Release from these systems depends on the drug concentration, since the rate of the process rises linearly with an increase in drug concentration. In the *in vitro* release study carried out in this work, the cumulative percentage released in all cases corresponds to the parameter estimated in the non-linear regression (% VEGF$_{released\ \infty}$ and % BMP-2$_{released\ \infty}$). With these results, we can conclude that complete release of the factors by diffusion has happened during the time of the assay. It is envisaged that the rest of the unreleased

protein will be released by the degradation of the scaffold under real physiological conditions (Yamamoto, *et al.* 2001). Actually, gelatin is considered as biodegradable material since, sensitive moieties to matrix metalloproteinases, the enzymes that remodel de extracellular matrix, are maintained in its structure (Cui, *et al.* 2017)

Additionally, we have observed that the total amount of all the collected samples does not fully coincide with the theoretical loaded dose. The difference in both proteins has been greater in the scaffolds GEL_20/20 (Figure 4A and Figure 5A). This could be due to the incorporation of collagenase at the end of the study to degrade the scaffold and quantify the remaining protein not released. It is well-known that collagenase is a protease that cleaves peptide bonds and therefore the structures of growth factors may be affected (Visse and Nagase 2003). In the GEL_20/20 scaffolds less amount of protein has been released during the assay and consequently the remaining fraction is expected to be higher. To ensure this effect, we have quantified the concentration of VEGF in PBS in the absence or presence of the same concentration of collagenase used in the *in vitro* release test. The results demonstrated that there is a significant decrease in the protein amount in the presence of this protease (Figure 6).

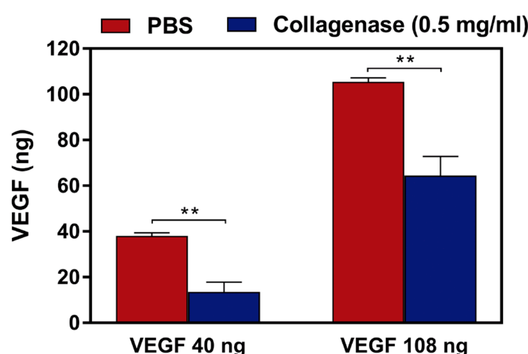


Figure 6. Amount of Vascular Endothelial Growth Factor (VEGF) detected by a commercial ELISA kit in two solutions: Phosphate Buffered Saline (PBS) and 0.5 mg/mL collagenase solution. The prepared solutions correspond to the theoretical estimated amount that would remain without release at the end of the study in the scaffolds: 40 ng of VEGF in GEL_10/20 scaffold and 108 ng of VEGF in GEL_20/20 scaffold. Statistical significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

The prototypes that have been developed in this work could be considered versatile release systems, since they present the potential to serve as a support for the release of various growth factors. However, the release profile is dependent on the ionic interaction with gelatin. For this reason, the release profile of both growth factors has been similar, delivering approximately half of the dose in the first 24 hours. The process of regeneration of the bone tissue is considered a set of events controlled spatio-temporally by the action of many

growth factors. For this reason, it could be immensely interesting to incorporate new design strategies to our systems to achieve the release of BMP-2 delayed over time, to better mimic the natural healing environment. The inclusion of osteoinductive growth factors inside of particulate systems of both micro and nano scale might help on tuning the release profile of such agents (Kim, *et al.* 2017).

4. CONCLUSIONS

In this experimental work, nine different 3D scaffolds using gelatin from bovine skin crosslinked with different ratios of mTG by freeze-drying technique, have been designed, produced and characterized. Porous structures have been obtained with a higher degree of swelling when the proportion of the enzyme was lower. The structures presented similar elasticity values of osteoid tissue. In all the prototypes absence of cytotoxicity was obtained. Two candidates were chosen to further study the cellular interaction, and capacity of retention and release of growth factors. The stemness and proliferative capacity of the MSCSs cultured on the scaffolds was reduced while the expression of osteoblast-related genes (*Col1a1, Runx2, Osx*) was potentiated. The selected prototypes were able to retain and release VEGF and BMP-2, following a first-order release kinetic. Our preliminary *in vitro* results suggest that gelatin-based 3D dual scaffolds could be useful for future *in vivo* bone regenerative purposes.

5. ACKNOWLEDGEMENTS

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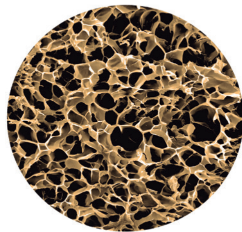
EXPERIMENTAL SECTION

CHAPTER 2



CHAPTER 2

Bioinspired gelatin/bioceramics composites loaded with bone morphogenetic protein-2 (BMP-2) promote osteoporotic bone repair

ABSTRACT

Bone defects healing results particularly challenging after a osteoporotic fracture, due to the poor osteogenic capacity of that tissue. The development of both bioactive and osteoconductive biomaterials that allow reducing the doses of bone morphogenetic protein – 2 (BMP-2) is one of the promising strategies to optimize regenerative therapies for these injuries. Herein, we developed gelatin-based 3D scaffolds reinforced with either calcium sulfate or hydroxyapatite as an osteoconductive biomaterial suitable for osteoporotic bone regeneration. The organic/inorganic composite systems showed excellent swelling capacity and good *in vitro* degradability. The incorporation of ceramic compounds resulted in stiffer scaffolds without comprising the cellular biocompatibility. The assessment of the biological commitment with human bone-marrow derived mesenchymal stem cells (hBM-MSCs) revealed adequate surface properties of the 3D scaffolds to promote cell adhesion and proliferation along with osteogenic differentiation capabilities. Specifically, downregulation of stemness (*Nanog, Oct4*) genes and upregulation of osteogenic markers (*ALP, Col1a1, Fmod*) were observed under basal culture conditions. Promisingly, the sustained *in vitro* release of BMP-2 observed from the porous reinforced scaffolds allowed us to address the critical-sized osteoporotic mice calvarial defects with a relatively low protein dose (600 ng BMP-2/scaffold). The *in vivo* results revealed that gelatin composite scaffolds functionalized with BMP-2 could significantly enhance bone formation within 8 weeks. Overall, this study demonstrates the promising potential of osteoconductive gelatin/calcium bioceramics composites as osteogenic growth factors delivery carriers, especially for osteoporotic bone defects approaches.

Keywords: bone, bone morphogenetic protein -2, calcium sulfate, gelatin, hydroxyapatite, osteoporosis.

1. INTRODUCTION

Osteoporosis is one of the most frequent bone disorders characterized by the decrease in the bone mass and the deterioration of the microstructure of bone tissue, which results in increased bone fragility and therefore, susceptibility to fractures (1). It is estimated that more than 9 million osteoporotic fractures occur every year worldwide. In adulthood the mass and volume of the bones remain constant because of the coordinated homeostasis of bone formation and resorption by osteoblast and osteoclasts, respectively. The excess of resorption against the bone formation due to the loss bone turnover regulation is behind the pathophysiology of this disease (2). The current osteoporosis treatment consists of systemic administration of antiresorptive drugs (e.g. bisphosphonates) that inhibit the function of osteoclasts and anabolic agents (e.g. teriparatide) that induce bone formation to maintain the bone mass. Importantly, it has been demonstrated that during the development of this pathology, bone marrow derived mesenchymal stem cells (BM-MSCs) reduce their ability to differentiate into osteoblasts and increase their differentiation to adipogenic lineage (3). Therefore, the poor osteogenic capacity due to the disruption of BM-MSCs differentiation balance makes the repair of bone defects after a osteoporotic fracture especially challenging.

The synergistic integration of therapeutically active growth factors and drug delivery platforms is one of the most promising approaches comprised within the regenerative medicine field. Regarding bone tissue healing, one of the most extensively studied osteoinductive growth factor is the bone morphogenetic protein – 2 (BMP-2), which belongs to the transforming growth factor β (TGF-β) superfamily (4). This pleiotropic cytokine induces the osteogenic differentiation of MSC and osteoprogenitor cells during the reparation of bone tissue (5). Due to the low bioavailability of this factor, immensely high doses of BMP-2 compared to physiological levels found in a fracture, have been necessary to achieve a therapeutic effect in the clinic. For instance, micrograms to tens of micrograms of BMP-2 in rodents and up to tens of milligrams in humans have been used. There are currently several commercialized products approved by the Food and Drug Administration and the European Medicines Agency based on the use of recombinant human BMP-2 for the treatment of non-unions long fractures and spinal fusion. However, the adverse effects recorded with the use of these medicines suggest the need for the development of new drug delivery carriers that allow reducing the required doses and improve the cost-effectiveness profile. The development of systems that are intrinsically more osteoconductive with reduced dependence on BMP-2 to promote bone regeneration would address these problems and improve the osteogenic capabilities within the bone tissue engineering field (6).

These carriers would ideally allow not only the controlled release of the growth factor to customize its exposure at the site of injury but provide the essential cues to promote the adhesion, proliferation and extracellular matrix deposition by host cells, which are already partially

differentiated (e.g. preosteoblasts). To achieve this goal, bioinspired organic/inorganic composite systems that resemble the natural composition of the bone tissue have extended been proposed (7,8).

Regarding the materials to create the biomimetic composite, natural origin gelatin is a promising candidate for the organic phase. The exceptional cell-responsive properties and intrinsic ability to create polyion complexes with charged therapeutic agents have made this biodegradable protein particularly fruitful biomaterial for biomedical applications (9). On the other hand, calcium phosphate and calcium sulfate-based bioactive ceramics own bone-bonding characteristics and their ability to stimulate bone formation could improve the osteoconductivity of the composite. Although these materials have been previously used as bone void fillers, the poor bioresorbability of calcium-phosphate hydroxyapatite (HA) in particular, difficulty for the stable surgical fixation of these stiff/brittle substitutes and the lack of vascularization in their central part have limited their use as sole material of the graft (10,11).

In the present study, we present a gelatin-based 3D scaffold tailored to support the osteogenic commitment and the release of osteogenic BMP-2 growth factor by incorporating calcium ceramic compounds for osteoporotic bone tissue engineering purposes. For this aim, the gelatin network was enzymatically crosslinked and the therapeutic agent was loaded to the preformed scaffolds. Different ratios of calcium sulfate and HA were successfully incorporated into the matrices and the swelling, degradation and mechanical properties were evaluated. Furthermore, the analysis of the microstructure and chemical composition were evaluated. The biological performance of the developed scaffolds was assessed by culturing human bone-marrow derived mesenchymal stem cells (hBM-MSCs) on the scaffold surface and cellular adhesion, proliferation, viability and osteogenic differentiation capacity were determined. hBM-MSCs differentiation fate was assessed through the analysis of the expression levels of both stemness (*Nanog, Oct4*) and osteoblast-related genes (*Col1a1, Runx2, Fmod*) by RT-PCR assay. Moreover, a proteomic platform was optimized for better understanding the cell-biomaterial interaction. Finally, bone regenerative potential of the developed 3D scaffolds was investigated in osteoporotic mice critical-size calvarial defects.

2. MATERIALS AND METHODS

2.1 Materials

Gelatin from bovine skin Type B (~ 225 g Bloom), calcium sulfate dihydrate, hydroxyapatite particles, Cell Counting Kit-8 (CCK-8), Triton X-100, collagenease P, bovine serum albumin (BSA), p-nitrophenyl phosphate (pNPP) and calf intestinal alkaline phosphatase (ALP) were purchased from Sigma Aldrich, Spain. Microbial Transglutaminase (100 U/g) was kindly supplied by Ajinomoto Foods Europe, France. Phosphate Buffered saline (PBS), Trypsin, fetal bovine serum (FBS), Penicillin-Streptomycin solution, Dulbecco's

Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), Pierce™ BCA Protein Assay Kit, 4,6-diamidino-2-phenyindole dilactate (DAPI), AlexaFluor 488-phalloidin, LIVE/DEAD® kit and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were obtained from Fisher Scientific, Spain. Calcium Detection Kit was purchased from Abcam and TRIsureTM reagent from Bioline. Recombinant human bone morphogenetic protein -2 (rh-BMP-2) was purchased from GenScript and ELISA kit for rh-BMP-2 detection from Peproptech, UK.

2.2 Fabrication of 3D scaffolds

Enzymatically crosslinked gelatin-based 3D scaffolds were prepared by the freeze-drying technique. In brief, 1 g of gelatin from bovine skin was dissolved in 3 mL distilled water under constant stirring at 40 °C for 1 hour. For the preparation of reinforced scaffolds, calcium sulfate salt or HA particles at different ratios were mixed with gelatin to obtain hydrogels with different grade of reinforcement: low, medium or high - 3.75, 7.5 and 15 % (w/v) reinforcing material, respectively. Then, gelatin dispersions with or without reinforcing materials were mixed in a 1.5:1 (v/v) ratio with the enzyme solution and briefly homogenized under magnetic stirring. Hence, the final scaffolds were composed of 20 % (w/v) gelatin and 20 U/g gelatin enzymatic activity was used to get fully crosslinked systems. Afterward, the hydrogels were casted into polystyrene molds and punched out to obtain desirable sized cylindrical 3D scaffolds. These structures were immersed in ethanol 70% (v/v) for 15 minutes and thereafter two washings with PBS were performed, in order to discard any remaining ethanol. Subsequently, the samples were first frozen at -80 °C, and freeze-dried after.

2.3 Swelling ratio and degradation profile

The swelling behavior of the gelatin-based scaffolds was determined in PBS at 37 °C under constant shaking (300 rpm). The initial dry weight of each sample was measured before their immersion in PBS. At the end of each period of time (1, 2, 5, 10, 15 and 30 min, 1, 2, 7, and 24 hours and 6 days) the wet weight of the samples was acquired, after the excess of the surface liquid was removed. The swelling ratio was calculated following equation 1, where Ws is the weight of the wet sample and the Wo corresponds to the initial dry weight of the same sample.

[Eq. 1] Swelling Ratio = (Ws – Wo)/Wo

To simulate the stability of the samples under physiological conditions, both hydrolytic and enzymatic degradation tests were performed. For the hydrolytic degradation test, the samples were first immersed in PBS until swelling equilibrium was reached (2 hours) and weighed then. This value was considered the initial weight and the samples were immersed in PBS and maintained at 37 °C during 9 days. At each time point, samples were taken out

and the weight of each sample was recorded. The degradation rate was determined by the weight of the remaining matrices. For the enzymatic degradation test, collagenase P solution at 0.02% (w/v) concentration was used to immerse the samples. In these case, when the samples were completely degraded the total protein and calcium content of the supernatant were determined by commercially available kits, following the instructions of the manufacturer.

2.4 Physicochemical characterization

2.4.1 Mechanical compressive properties

The compressive mechanical behavior of the developed scaffolds was analyzed by static uniaxial unconfined and confined compression test following the protocol described previously (12), with minor variations. An universal mechanical testing equipment (Instron 5548) equipped with 50 N load cell, under a compression rate of 1 mm/min was used. The Young and Aggregate moduli were determined from the slope of the stress-strain curve in the 10-20% strain linear region from the unconfined and confined compression tests, respectively. The Poisson coefficient (v) for each type of scaffold was determined following the equation 2, where Es is the value of the Young modulus and HA corresponds to the Aggregate modulus.

[Eq. 2] $Es= [((1+v)(1-2v))/(1-v)]\ HA$

2.4.2 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDAX) Analysis

In order to determine the porosity of the hydrogels SEM analysis were performed. SEM images were acquired with SEM Quanta FEG 250 Analytical ESEM instrument operating at an accelerating voltage 2 kV in high vacuum mode after gold sputtering the freeze dried hydrogels with 5 nm. ImageJ software was used to measure the pore size of the collected SEM images and average pore size was calculated from at least 40 measurements from each sample. EDAX analysis was performed with Oxford Instruments 80 mm^2 X-Max silicon drift detector connected to the SEM instrument after gold sputtering with 5 nm.

2.4.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The chemical composition of the lyophilized scaffolds was assessed by means of FTIR spectroscopy. FTIR spectra of the freeze-dried hydrogels were recorded on attenuated total reflection (ATR) mode using a PerkinElmer Spectrum 100 FTIR spectrophotometer with an ATR accessory after background subtraction. Spectra were recorded over the range of 4000-500 cm^{-1} with 16 scans at a resolution of 4 cm^{-1}. The collected spectra were baseline-corrected and normalized using PerkinElmer Spectrum software and the average of four spectra were used for the analysis.

2.4.4 X-ray Diffraction (XRD) Analysis

The XRD patterns of the freeze dried hydrogels were collected with a Huber G670 powder diffractometer in the 2θ range of 3 to 100° in steps of 0.005° using CuKα1 radiation (λ = 1.54056 Å) for 10 min. The data were collected in transmission mode from a rotating flat plate sample inclined 45° relative the primary beam.

2.5 *In vitro* release of BMP-2

The *in vitro* release of BMP-2 growth factor from the developed formulations was evaluated with 4 mm diameter scaffolds and Protein Lobind Eppendorf tubes. First, the 3D scaffolds were loaded with 600 ng of BMP-2 and they were incubated overnight for the protein adsorption. Afterward, a washing step with 1 mL of PBS was performed to remove the unbound protein fraction before the sampling phase was initiated. The *in vitro* release assay was performed at 37 °C under mild orbital agitation and at each time point, all the volume of PBS in tubes was collected and replaced with fresh one. After collecting the last sample, the scaffolds were degraded with collagenase P solution in order to determine the amount of growth factor remaining in the scaffolds. All the samples were kept frozen until the determination of the BMP-2 concentration by commercial ELISA kit following the manufacturer´s guideline.

2.6 Biocompatibility study

The preliminary cellular compatibility of the scaffolds was assessed as described by Echave MC *et al.* (13), following the guideline ISO 10993 (Biological evaluation of medical devices guideline: cytotoxicity on extracts and cytotoxicity by direct contact). Both the cytotoxicity produced by cell-scaffold direct interaction and the indirect toxicity of the scaffolds were determined by the evaluation of the cells metabolic activity trough the CCK-8 assay. The metabolic activity of cells without contact with scaffolds or the extracts was considered as 100% of viability.

2.7 *In vitro* cell studies: human Bone-Marrow derived Mesenchymal Stem Cells (hBM-MSCs) seeding on the developed systems

2.7.1 hBM-MSC expansion and seeding

hBM-MSC were expanded in complete basal medium consisting of DMEM/F12 culture medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and the split of the cells was done when reached confluence. hBM-MSCs were used at passage 3-5 for all the cellular studies. Before the seeding of the cells on the top of the 3D systems, all the scaffolds were exposed to UV light for 15 minutes and hydrated with the basal culture medium. The hydrated scaffolds were placed in wells of the same size in ultra-low attachment microplates (Corning™ Costar™ Ultra-Low Attachment Microplates) and 10^5 hBM-MSCs/scaffold in 30 µL of medium

were seeded onto the surface of the scaffolds. These scaffolds were kept in the CO_2 incubator for 1.5 hours and then 1.5 mL of complete basal medium was added to each well. The culture medium was changed every 2-3 days during the experiments.

2.7.2 Live/dead viability assay

Cell viability at the enzymatically crosslinked gelatin-based substrates was evaluated using Live/Dead viability assay (Life Technologies) according to the manufacturer´s protocol. After 2 and 10 days of culture, three samples of each group (GEL, $CaSO_4$_7 and HA_7) were incubated in calcein-AM/ethidium solution for 30 min and fluorescence micrographs were taken using inverted fluorescence microscope (Nikon TMS). Flow cytometry (MACSQuant Analyzer, Miltenyi Biotec) was used to quantify the viability of the cells seeded on the surface of the scaffolds at day 10. First, cells were detached from the scaffolds with trypsin-EDTA and dyed after with Live/Dead kit, following the guidelines.

2.7.3 Cell adhesion, proliferation and metabolic activity determination

Immunofluorescence staining was performed to study the adhesion and proliferation of hBM-MSCs on the scaffolds. The cell-seeded scaffolds were washed with PBS and fixed with 3.7% (v/v) formaldehyde for 10 min after 7 days of culture. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and the blocking of the samples was performed with 1% (w/v) BSA for 30 min. The F-actin filaments were stained with 165 nM AlexaFluor 488-labelled phalloidin for 30 min at room temperature protected from light. After washing the samples with PBS three times, the nuclei were stained with 300 nM DAPI solution. The samples were observed under inverted fluorescence microscope (Nikon TMS).

The proliferation of hBM-MSCs on the 3D scaffolds was evaluated by means of metabolic activity determination following CCK-8 assay. The cell-seeded samples were rinsed with PBS after 2, 7 and 10 days of incubation and 350 µL of fresh medium containing 35 µL of CCK-8 kit reagent was added to each scaffold. The samples were incubated for 4 h at 37 °C and the optical density of the generated formazan was measured by means of Tecan Infinite M2000 microplate reader.

2.7.4 Alkaline Phosphatase (ALP) activity and staining

The secretory form of ALP from the cells was evaluated spectrophotometrically during 3 weeks. The activity was evaluated by determining the hydrolysis of p-nitrophenyl phosphate by ALP at pH 9.3. A standard curve with calf intestinal ALP was prepared and 100 µL of pNPP at 0.2% (w/v) was added to each sample. The reaction was stopped with 50 µL of NaOH 3M and absorbance measured at 405 nm. Moreover, for the evaluation of the intracellular ALP activity of hBM-MSC seeded onto the scaffolds at the end of the assay, BCIP/NBT solution was used. After washing the scaffolds three times with PBS, the samples were covered with the solution and incubated protected from light at room temperature for 2 h. Then, the excess

of the dye was discarded washing the scaffolds three times with PBS. The stained scaffolds were observed under bright field and imaged with a digital camera. Image J software was used for the analysis of the images and the area covered by the black-violet stained cells was determined by applying a threshold. The entire area of the scaffold was considered as 100%.

2.7.5 RNA Isolation and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (q-RT-PCR)

Total RNA was isolated from primary hBM-MSCs using TRIsure reagent (Bioline, London, UK). 1 µg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using the SensiFAST Probe Hi-ROX Mix (Bioline) in an ABI Prism 7900 HT Fast Real-Time thermocycler. The genes were screened using Taqman 5´-nuclease probe method (Applied Biosystems) and all transcripts were normalized to the housekeeping gene TATA binding protein (*Tbp*). Fold-change expression was calculated using the $2^{-\Delta\Delta Ct}$.

2.7.6 Proteomic analysis

The optimization of the sample processing and analysis methodology has been carried out to determine the proteomic profile of the cells seeded on the developed scaffolds. For that purpose, cell culture conditions, protein elution from 3D scaffolds and the detection technology through mass spectrometry have been evaluated.

Briefly, 10^5 hBM-MSCs were seeded on the surface of each scaffold, previously hydrated in culture medium, as detailed above. These cells were maintained in *in vitro* culture for 10 days, changing the culture medium every two days. After this period, the samples were processed for the cellular lysis and the protein extraction. The eluted proteins were digested in solution, following the filter-aided sample preparation (FASP) protocol described previously by Wisnewski *et al.*, with minor modifications (14) . The resulting peptides were loaded onto LTQ Orbitrap XL-ETD (Thermo Fisher Scientific) or timsTOF Pro (Bruker) mass spectrometers. For the differential protein analysis, Progenesis (Nonlinear Dynamics, Newcastle, UK) or PEAKS® software were used, and the preliminary functional annotation of the proteins was evaluated using DAVID GO annotation programme (https://david.ncifcrf.gov/).

2.8 *In vivo* evaluation of bone regeneration

2.8.1 Osteoporotic mice calvaria defect model

The animal experiments were carried out in conformity with the European Directive (2010/63/UE) on Care and Use of Animals in Experimental Procedures. In addition, the animal protocols were previously approved by the Ethics Committee for Animal Care of the University of La Laguna. The surgeries were carried out under isoflurane anesthesia. The analgesia consisted in buprenorphine (0.01 mg/kg) by subcutaneous route before the surgeries

and paracetamol (200 mg/kg) in the drinking water, for 3 days post-surgery. Furthermore, after recovery from the surgeries, animals were allowed free movement, food and water uptake. Experimental osteoporosis was induced to 20 female FVB mice, approximately 16 weeks old, by bilateral ovariectomy, via dorsal approach. Immediately after the surgery the mice received 3 mg/kg body weight of dexamethasone-21-isonicotinate (Deyanil retard, Fatro Ibérica, Spain) administered subcutaneously once a weeks for up to 16 weeks. Then, the animals underwent a new surgery to create the bone defect and simultaneously the scaffolds with the treatments to be tested, were implanted. Briefly, the calvaria bone was exposed and a 4mm circular area was delimited with a biopsy punch. Then, a 4 mm circular trans-osseous defect was made with a trephine bur (15). The scaffold was inserted in the defect and the skin was stapled. At 8 weeks post-implantation, animals were sacrificed by CO_2 inhalation and the defect area was extracted.

2.8.2 Histology, histomorphometry and immunohistochemistry analysis

To determine the capacity of the implanted scaffolds to regenerate the critical size defect practiced in the calvaria of the mice, the extracted samples were prepared for histological analysis as previously described (16). Briefly, samples were fixed in paraformaldehyde 4% solution first, decalcified in Histofix® Decalcifier (Panreac, Barcelona, Spain) and dehydrated in a graded series of ethanol after, before they were embedded in ParaplastVR. Longitudinal microtome (Shandon Finesse 325) sections with 3–5 μm thickness were prepared throughout the defect site. The sections were stained with hematoxylin-erythrosin for new bone formation visualization. Bone mineralization was assessed with VOF trichrome staining, in which red and brown staining indicates advanced mineralization, whereas less mineralized, newly formed bone stains in blue (17). Sections were analyzed by light microscopy (LEICA DM 4000B) and computer based image analysis software (Leica Q-win V3 Pro-image Analysis System, Barcelona, Spain) was used to evaluate all sections. A region of interest (ROI) within the defect (12.5 mm^2) was defined for quantitative evaluation of new bone formation. New bone formation was expressed as a percentage of repair with respect to the original defect area within the ROI.

For immunohistochemical analysis, sections were deparaffined and rehydrated in Tris-buffered saline (TBS) (pH 7.4, 0.01 M Trizma base, 0.04 M Tris hydrochloride, 0.15 M NaCl), which was used for all further incubations and rinse steps. Sections were incubated in citrate buffer (pH 6) at 90 °C for antigen retrieval, followed by incubation in 0.3% hydrogen peroxide in TBS buffer for 20 min. After a rinse step, sections were blocked with 2% FBS in TBS–0.2% Triton X-100 (blocking buffer). The indirect immunohistochemical procedure was carried out by incubating the sections with osteocalcin (OCN) antiserum (1/100) (Millipore, Barcelona, Spain) in blocking buffer overnight at 4 °C. Sections were rinsed three times, then incubated with biotin-SP-conjugated donkey anti-rabbit F fragment (1/500)

(Millipore, Barcelona, Spain) in blocking buffer for 1 h followed, after another rinse step, by incubation in peroxidase-conjugated streptavidin (1/500) (Millipore, Barcelona, Spain) for 1 h. Peroxidase activity was revealed in Tris–HCl buffer (0.05 M, pH 7.6) containing 0.005% of 3.3' diaminobencidine (Sigma, Poole, UK) and 0.01% hydrogen peroxide. Reaction specificity was confirmed by replacing the specific antiserum with normal serum or by pre-adsorption of the specific antiserum with the corresponding antigen. OCN staining was evaluated using computer-based image analysis software (ImageJ, NIH, Bethesda, MD). OCN staining was measured by applying a fixed threshold to select for positive staining within the ROI. Positive pixel areas were divided by the total surface size (mm^2) of the ROI. Values were normalized to those measured from blank scaffolds and are reported as relative staining intensities.

2.9 Statistical analysis

The statistical analysis of the data was completed using GraphPad PRISM (7.0) software. The normal distribution of the data was checked by the Shapiro-Wilk test. For normally distributed data, Student´s *t*-test or one-way ANOVA were applied for differences between two groups or multiple comparisons, respectively. Tukey *post-hoc* test was applied for multiple comparisons. For non-normally distributed data, Mann-Whitney nonparametric analysis or Kruskal-Wallis test with Dunn´s multiple comparisons tests were applied. In all cases, $p < 0.05$ values were considered as significant, represented by symbols described in the graphs. Data are presented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Swelling capacity and degradation profile

The development of organic/inorganic composite 3D scaffolds was driven through the freeze-drying technique. The organic part consisted of enzymatically crosslinked gelatin network, whereas two types of bioceramics were evaluated for the inorganic phase. Thus, the gradual incorporation of HA and calcium sulfate into the polymeric hydrogel resulted in three levels of reinforcement and seven different types of formulations. First, we characterized the main properties of all prototypes and then preselect only the best candidates for further *in vitro* and *in vivo* assessment (Figure 1A).

Regarding the water uptake process by dried scaffolds, all the formulations showed great ability to swell. Although the incorporation of fillers did not affect the kinetics of such a process (all the samples were swollen after 1 h), the swelling ratio decreased proportionally with the level of reinforcement (Figure 1B). These results are in accordance with those presented recently by others in which the swelling ratio of gelatin/HA cryogels decreased with the increase of HA concentration (18,19). Nevertheless, the values of swelling ratio of the reinforced scaffolds were still within the range previously defined for tissue engineering applications (20).

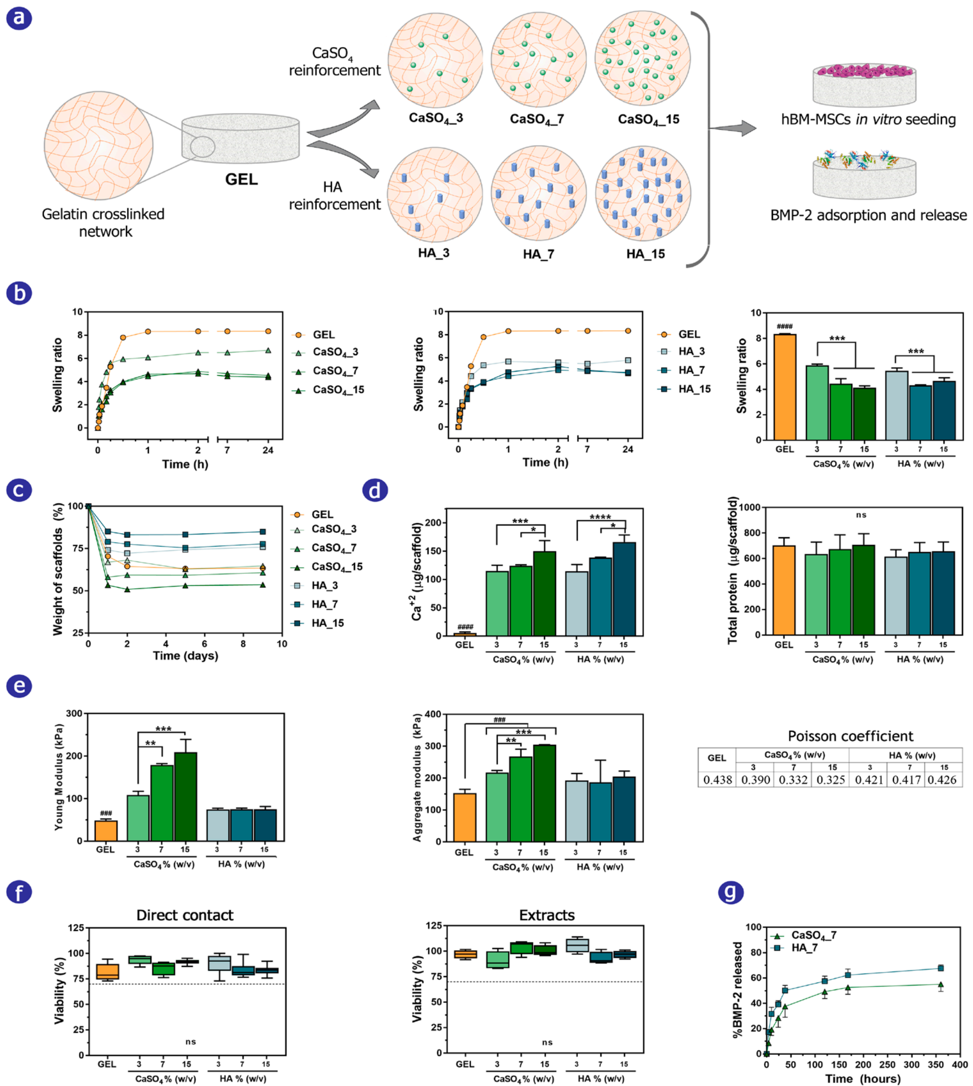


Figure 1. Development and characterization of reinforced gelatin-based scaffolds. (a) Schematic illustration of the design strategy of gradually reinforced gelatin scaffolds with either $CaSO_4$ or HA and the *in vitro* biological evaluation with hBM-MSCs seeding and BMP-2 growth factor delivery. (b) Determination of swelling properties of the systems. *In vitro* hydrolytic (c) and enzymatic (d,e) degradation of the 3D scaffolds. (e) Compressive mechanical properties of the developed systems. (f) Biocompatibility properties assessed by extracts and direct contact cell-toxicity assays. (g) *In vitro* cumulative release of BMP-2 from the selected scaffolds. Statistical significance: ns = no significant, * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001; ### p<0.001 and #### p<0.0001 compared GEL to all the reinforced scaffolds.

3.2 Mechanical properties determination

Uniaxial confined and unconfined static compression tests were performed to evaluate the mechanical properties of the composite scaffolds and Young and Aggregate modulus together with Poisson coefficient were determined for each sample (Figure 1E). The Young modulus, which is representative of material elasticity, was increased by means of the incorporation of inorganic components into gelatin-based organic network ($p< 0.001$). In the case of calcium sulfate containing scaffolds, this parameter increased significantly along with the concentration of the mineral. Thus, we found a 2-fold increase in Young modulus for $CaSO_4$_15 (209 ± 30 kPa) compared to $CaSO_4$_3 (108 ± 9 kPa). Conversely, although the incorporation of HA resulted in a reinforcing effect compared to the GEL scaffold, no differences were observed between the different prototypes. On the other hand, the Aggregate modulus determined from the confined tests were higher than the Young modulus in all the prototypes, suggesting that scaffolds generated a lateral expansion during unconfined axial compression (26). Therefore, the Poisson coefficient was above 0 for all the samples.

Interestingly, some studies have demonstrated that hydrogels with a Young modulus similar to our HA-reinforced scaffolds (~ 60 kPa) induce optimal MSC osteogenesis both *in vitro* and *in vivo* (27). However, the higher values of calcium sulfate-reinforced scaffolds may also advantageous, particularly during the surgical insertion. In fact, although the bulk mechanical requirements for osteogenic 3D scaffolds designed to serve as resorbable temporary structures differ from those weight-bearing dense permanent implants, swollen 3D porous scaffolds must present enough stiffness to ensure proper surgical handling and stable graft fixation. In this regard, Zhang B. *et al.* have recently achieved exceptional results regarding the stable fixation rate, with multifunctional amphiphilic copolymer/HA composite grafts presenting compressive modulus of 126-181 kPa after scaffolds hydration process (28).

Therefore, considering the preliminary examined physical and chemical properties, we limited the further characterization, biological commitment evaluation and *in vivo* bone regeneration performance assessment to scaffolds composited of 7% of calcium mineral elements and GEL scaffold as control group.

3.3 Microstructure and elemental composition characterization

The microstructure analysis of the 3D scaffolds was performed by SEM. As shown in the representative SEM images depicted in Figure 2A, the scaffolds were porous. The pore size of the scaffolds is an important feature which influences the cell-cell interaction, cell migration, proliferation and differentiation processes during the regeneration of the tissue. In the case of the developed scaffolds, the incorporation of ceramic compounds increased the size of pores, especially with HA inclusion. According to some previous studies, 3D scaffolds intended for bone tissue engineering with a pore size of around 100 μm favored the

migration and proliferation of osteoblast, thus promoting the bone formation (29). Moreover, an efficient oxygen diffusion and waste and nutrient exchange between infiltrated cells and the surrounding environment is expected with this range of pore size (30).

To examine the effective incorporation of osteoconductive minerals into the 3D gelatin structure, the evaluation of chemical structure was performed by means of EDAX mapping, FTIR and XRD analysis. Regarding EDAX measurements Figure 2B), uniform distribution of calcium was noticed in both composite scaffolds. As expected, sulfur and phosphorous elements were observed in $CaSO_4$_7 and HA_7 composite scaffolds, respectively. The presence of reinforcing minerals was confirmed via the FTIR spectra. Infrared spectra of raw materials and the scaffolds are shown in Figure 2C. The characteristic bands of gelatin in 1023 (C-O-C stretching), 1342 (Amide III) and 3289 (Amine peak) cm^{-1} were observed in all the scaffolds. In the case of $CaSO_4$_7 scaffold, the amide I peak of gelatin shifted from 1634 to 1622 cm^{-1}. In addition, characteristic bands of the sulfate groups (SO_4^{-2}) were observed in this composite scaffold: 1112 (ν4 antisymmetric stretch vibration) and 596, 665 cm^{-1} (ν4 antisymmetric bending vibration). On the other hand, regarding the spectra of scaffold reinforced with HA, the characteristic Amide II peak of gelatin shifted from 1535 to 1543 cm^{-1}. Moreover, the absorption bands at 601, 558 cm^{-1} (ν4 bending vibration of phosphate groups) and 1029 cm^{-1} (ν3 streching vibration of phosphate groups) confirmed the presence of HA within the developed composite scaffold. The functional incorporation of both reinforcing calcium minerals was further confirmed with XRD analysis (Figure 2D), since HA and calcium sulfate corresponding peaks were recorded in respective composites. Interestingly, the crystalline structure of HA and calcium sulfate remained the same after the composite formation.

3.4 *In vitro* release of BMP-2

The release profile of bioactive BMP-2 factor from the organic/inorganic composite scaffolds (Figure 1G) demonstrated that the developed biomaterials facilitate the release of the osteogenic growth factor over an extended period. The first burst release of the protein from the scaffolds was within the first 2 days and just 35-50% of the loaded BMP-2 was delivered in that period. Following that phase, the release of the growth factor was maintained from both gelatin scaffolds reinforced either with calcium sulfate or HA particles and around the 50 and 60% of the loaded dose was delivered until the end of the assay, respectively.

Currently, the spatiotemporal control over the presentation of therapeutic factors at the injured site is a major challenge associated to the growth factors based therapies for tissue regeneration. Due to the short half-life of these biological factors, the use of supraphysiological doses to achieve the desired therapeutic effect is inevitably accompanied by several adverse effects that limit the consolidation and expansion of these therapies in the usual clinical practice (31). For instance, ectopic bone formation, renal complications and cytotoxic effects have

been broadly observed with the use of INFUSE® bone graft clinically approved for lumbar spinal fusion promotion (32). Even though more advanced and complex systems with growth factors encapsulated in micro and nanoparticles as well as gene-therapy approaches (33,34) are being investigated to improve the spatiotemporal control of the therapeutic agents, it is expected that the bioactive constructs developed through the simple soak loading procedure may improve the strict regulatory concerns regarding the clinical translation (6). Therefore, we hypothesize that enhancing the osteoconductive properties of the drug carrier by means of adding calcium components to the formulation could permit the reduction of the required dose of the BMP-2 for the therapeutic effect and relieve the side effects associated with high dosing levels.

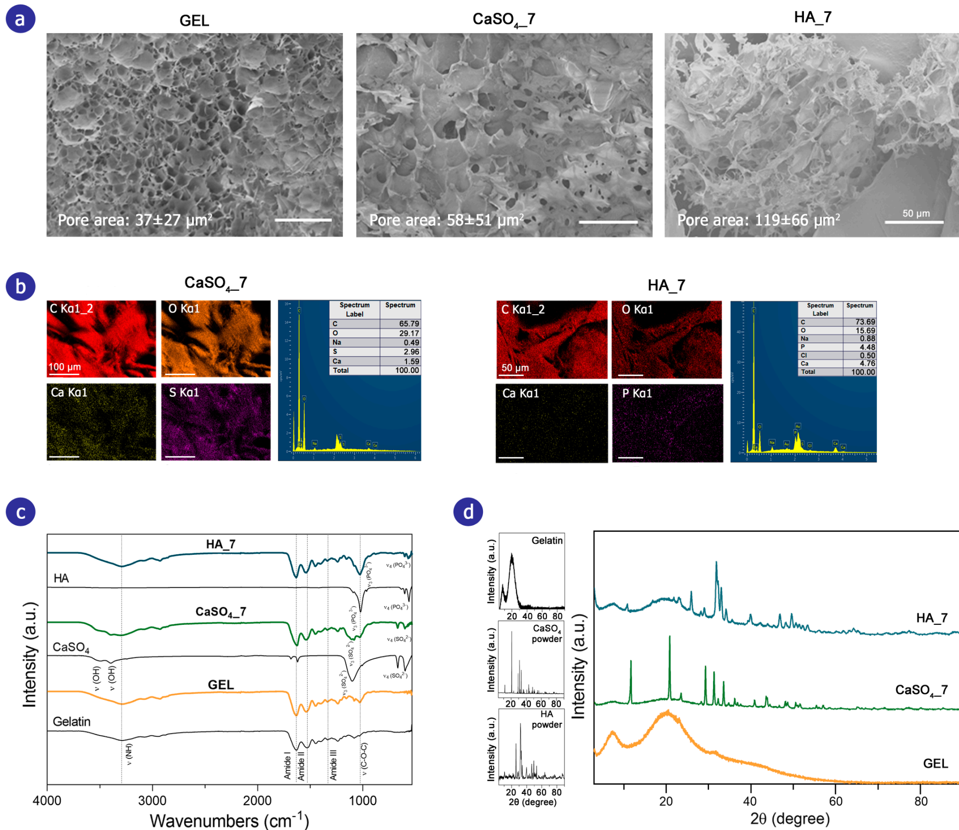


Figure 2. Morphological and chemical composition characterization of the gelatin-based composite scaffolds. (a) Representative SEM images of lyophilized scaffolds. (b) EDAX elementary mapping and the corresponding spectrum of the osteoconductive mineral composites. FTIR spectra (c) and XRD patterns (d) of pristine materials and developed gelatin-based composite scaffolds.

Importantly we have previously demonstrated that the scaffold GEL suggested as a control in this experimental work has the capacity to promote the sustained release of soak-loaded BMP-2 and the angiogenic factor VEGF following the first order kinetic. In that case, the burst release of approximately 50% VEGF and 40% BMP-2 was evident within the first day (13). Hence, the incorporation of reinforcing components does not affect the innate ability of the gelatin to guide the prolonged release of biological agents. Moreover, a slight extension of the burst effect over time was noticed, probably because of the non-specific electrostatic interactions occurred between the anionic groups of the sulfates and phosphate salts and the cationic groups of the growth factor (35,36).

3.5 Cell adhesion, proliferation and viability

First, the potential *in vitro* cytotoxic effect of implantable scaffolds was evaluated through indirect test with extracts and direct contact assay, using L929 fibroblasts as cell model. All experimental values exceeded the threshold limit established for non-cytotoxic biomaterials according to ISO10993 guidelines (Figure 1F). Therefore, unlike others calcium-phosphate based biomaterials (37), the range of bioceramics used in this work (3-15 % (w/v)) did not reveal any toxic effect on the cells due to the plausible ionic changes in the culture medium.

Moreover, the ability of selected prototypes to support the cell adhesion and proliferation was evaluated with hBM-MSCs seeding on their surface. The use of these primary cells to assess the *in vitro* biological performance and to determine the osteoinductive properties of 3D scaffolds is broadly studied because of their easy accessibility, high osteogenic differentiation ability and the elevated replicative potential (38). In addition, many efforts are being made to develop autologous bone grafts with cells isolated from the same patient (39,40). Regarding the viability of cells seeded onto the surface of the scaffolds evaluated by a live/dead assay, high rates of viability were achieved in all the samples (Figure 3A). Above 80% of the cells were alive after 10 days of culture and there were no significant differences between the groups (Figure 3B). These anchorage-dependent cells need to adhere to the surfaces to maintain their viability and osteogenic potential (41).

The fluorescent staining of the cytoskeleton and nuclei showed that the cells were completely adhered and spread on the surface of the scaffolds (Figure 3C). The mechanism beyond this adhesion may be mediated by the interaction between the integrin receptors present in cell membranes and the RGD motifs of the gelatin (42).

Furthermore, apart from the cellular adhesion produced on the surface of the scaffold, the cells proliferated during the *in vitro* culture as suggested by the increase in metabolic activity recorded between days 2 and 7 (Figure 3D). The cells could have reached confluence in a week, since the values of the CCK-8 assay did not increase from that moment (43). However, no significant differences on proliferation rates among the scaffolds were detected throughout

the entire period, suggesting that suitable platforms have been developed for osteoprogenitor cells to appropriately perform their role in the healing process.

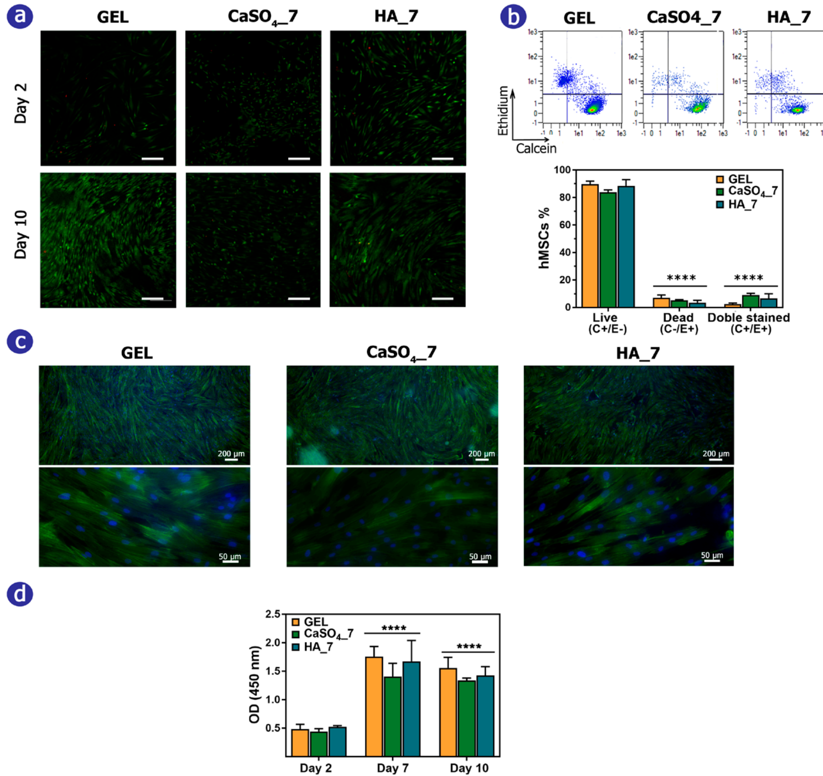


Figure 3. Adhesion, proliferation and viability of human bone-marrow derived mesenchymal stem cells (hBM-MSCs) seeded onto gelatin scaffolds. (a) Representative images of hBM-MSCs cultured onto the scaffolds for 2 and 10 days and Live/Dead staining. Scale bars = 500 µm (b) Quantification of cellular viability in different scaffolds after 10 days of culture analyzed by flow cytometry. (c) Fluorescence microscopy images of hBM-MSCs stained for nuclei (blue) and F-actin (green) after 7 days of culture on the scaffolds. (e) Metabolic activity of the cells cultured on the scaffolds for 2-10 days. Statistical differences: **** $p<0.0001$.

3.6 Assessment of osteogenic commitment

The evaluation of *in vitro* osteogenic differentiation potential of hBM-MSCs seeded on developed 3D scaffolds relied on the determination of ALP activity and the expression levels of two stemness (*Nanog, Oct4*) and three osteogenic (*Col1a1, Runx2, Fmod*) marker genes. First, the secreted ALP activity of cells was quantified for 3 weeks (Figure 4A). The ALP activity of the cells seeded in the bare gelatin scaffold (GEL) remained constant throughout the culture period, while a constant increase was determined in the case of the organic/inorganic composite scaffolds. In addition, this increase was more noticeable for scaffolds formulated with

HA. These differences were further contrasted with the qualitative chromogenic detection of this phosphatase at the end of the assay (Figure 4B). In the case of HA_7, the 50% of the scaffold surface was positively stained, while the covered area in the case of GEL was less than 10%. These results may suggest that the incorporation of the calcium elements to the gelatin scaffold could potentially enhance the osteoconductive properties of the formulation and promote the osteogenic commitment of host stem cells during the healing process. In fact, the ALP is an early bone marker protein involved directly in the process of the biomineralization. ALP hydrolyzes the pyrophosphate to generate inorganic phosphate, which is deposited in the form of HA when the accumulation of calcium and phosphate ions exceeds their solubility (44).

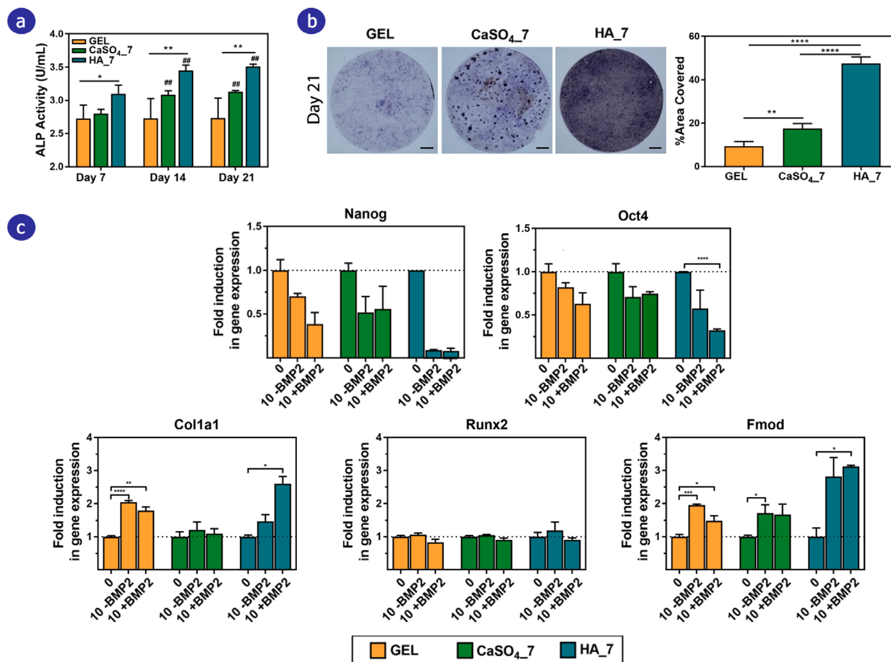


Figure 4. Evaluation of human bone-marrow derived mesenchymal stem cells (hBM-MSCs) osteogenic commitment after cultured on the developed scaffolds. (a) Determination of alkaline phosphatase (ALP) activity secreted by hBM-MSCs after 7, 14 and 21 days of culture. (b) ALP staining images and the relative quantification of the stained area after 21 days of cell culture. Scale bars = 1.5 mm. (c) The expression level of five selected gene markers at 10 days after incubation. The expression levels of all genes were normalized to that of *Tbp* as the housekeeping gene. Statistical differences: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; ## $p<0.01$ compared with the same scaffold at day 7.

The analysis of the expression level of several marker genes allowed us to better understand the state of the cell differentiation. The results from the RT-PCR assay are shown in Figure 4C and the changes in the genes expression suggested that cells seeded on the scaffolds were evolved trough differentiation processes. For instance, the expression levels of stemness genes (*Nanog, Oct4*) were downregulated over the time for all the scaffolds. On the other hand, a dissimilar pattern was observed for genes related to osteogenic differentiation. While the expressions of *Col1a1* and *Fmod* were upregulated during the course of the culture, the expression of *Runx2* remained stable. In addition, these results were in accordance with the ALP determination assay, because the increase showed in the expression of some osteogenic marker genes was more notable for scaffolds containing HA. Interestingly, the changes produced in the fate of the cells were caused only by the composition and structure of the scaffolds, since basal culture medium without any osteogenic supplementation was used for these experiments. In fact, external physical and chemical factors are considered to modulate the intracellular signaling pathways responsible for the orchestration of the differentiation of MSCs.

Although the transcription factor *Runx2* is considered as the main regulator in osteogenic differentiation through several signaling pathways such as canonical and non-canonical BMP, Wnt/β-catenin or ERK1/2 signaling pathways (45,46), researchers still exploring other signaling routes and molecular mechanism involved in the regulation of the osteogenic differentiation (47,48). For instance, some recent studies have concluded that the direct interaction between Osterix transcription factor and NFATc1 cofactor can promote the activation of the *Col1a1* gene promoter without activating the Runx2-dependent transcription (49,50). Thus, because of the effects of calcineurin-NFAT pathway has on the bone, potential therapeutic targets of this pathway are being considered for the development of new anabolic drugs for the treatment of osteoporosis. Recently Huang Y. *et al* demonstrated that the activation of this signaling pathway plays an important role in the anabolic effect of resveratrol on osteoblasts. For this reason, it seems necessary to extend the study of the cellular fate to conclude with more certainty the direction of the differentiation process and to describe the interactions between the hBM-MSCs and the developed scaffolds (51-53).

3.7 Optimization process of the conditions for proteomic analysis

In-depth knowledge about the interactions between developed biomaterials and biological systems such as, cells, tissues or organs, results in a striking strategy to guide the design of future advanced materials with improved functions. Exploring the actions carried out by proteins in these interactions could be particularly helpful to build the map of the cellular and molecular events involved there. In this context, nano scale liquid chromatography coupled on-line to tandem mass spectrometry (nLC MS/MS) is a powerful tool for the large-scale characterization of proteins offering the global and integrated evaluation of the entire proteome (54).

As an optimization process for the potential application of this analysis in order to further recognize the interactions between hBM-MSCs and the 3D scaffolds, specific variables classified in different categories (*in vitro* cell culture, extraction of proteins and detection of peptides) have been tested. A schematic representation of the workflow and the variables tested in each step are presented in Figure 5A.

For the first attempt, GEL and $CaSO_4$_7 scaffolds were chosen and comparison between empty scaffolds and scaffolds with seeded cells was carried out, in order to evaluate the background from the gelatin-based scaffold itself. These cells were cultured with standard basal medium containing 10% (v/v) FBS as in the other cellular experiments. The first step for the protein extraction was washing the scaffolds three times with PBS for 5 minutes, discarding proteins from the serum that might be adsorbed to the matrix and to promote the release of the possible remnant of culture medium within the system.

Consequently, cell lysis with cell lysis buffer (CLB) consisting on urea 7M, thiourea 2M and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) 4% was proposed as protein extraction protocol (55). Due to the possible cleavage of the disulfide bonds present in the gelatinous matrix, the lysis buffer was used without the reducing agent dithiothreitol (DTT). Nevertheless, in order to evaluate the extraction efficiency of cellular proteins against the background of bovine proteins, some of the samples were mixed for 1 minute with a vortex mixer and discarded the supernatant before adding the CLB. The extracted proteins were digested following the FASP method and the resulted tryptic peptides were analyzed by LTQ Orbitrap XL-ETD mass spectrometer. The results of this assay (Figure 5B-C) demonstrated that although human origin proteins could be identified, the background of bovine proteins was very high (about 50% of identified proteins). More interestingly, it was concluded that the proposed extra washing step carried out by vortex mixing was inappropriate for these samples. In fact, although the amounts of bovine proteins were not significantly diminished, a notable loss of human origin proteins was recorded (Figure 5C). The loss of cell adhesion to the scaffold due to the mechanical forces applied during the mixing may be behind this fact.

Subsequently, in order to improve the ratio of identified human/bovine proteins and increase the total number of identified proteins for each sample, modifications during *in vitro* cell culture and the data acquisition with last generation timsTOF Pro mass spectrometer were proposed as alternatives. Regarding the cellular culture conditions, two protocols with different culture medium were assessed. On the one hand, cells were cultured with standard culture medium until the ninth day and then, the medium was replaced by one containing only 1% FBS (mDMEM group). This strategy of reducing the concentration of the FBS has been previously described for another's biological experiments (56) and no remarkable change in the metabolic activity of the cells was noticed (Figure 6).

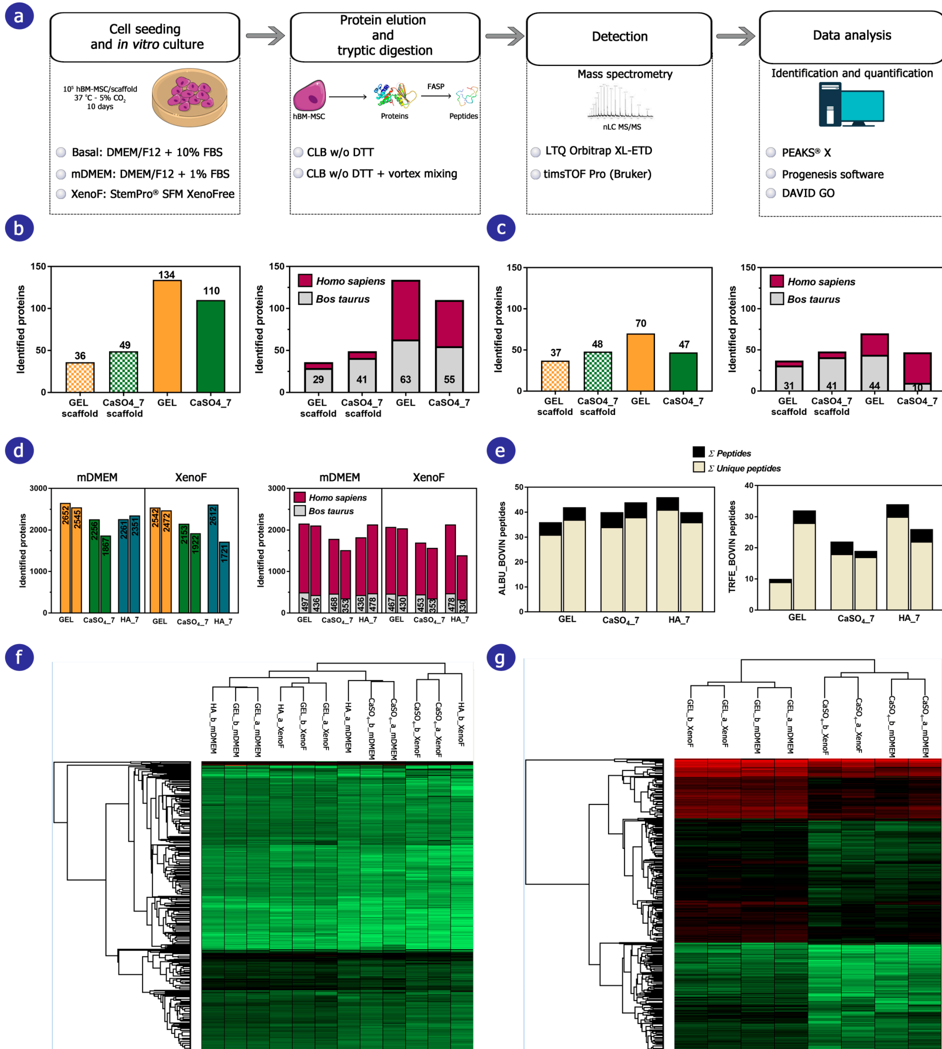


Figure 5. Evaluation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) proteomic profile by means of mass spectrometry after cultured on the developed scaffolds. (a) Schematic representation of the workflow for the optimization of conditions. (b) The total number of proteins identified and the distribution according to the organism of origin from cell lysates analyzed by LTQ Orbitrap XL-ETD mass spectrometer. (c) The effect of the extra washing step carried out by vortex mixing on the efficiency of protein elution. (d) The total number of proteins identified and the distribution according to the organism of origin from cell cultured with modified standard basal culture medium (mDMEM) or serum-free commercial culture medium (XenoF) analyzed by timsTOF Pro mass spectrometer. (e) The proportion of the unique peptides identified in XenoF group samples for bovin albumin and serotransferrin proteins. (f) Proteomic profile comparison between cells seeded on the developed scaffolds regarding the intensities assigned to each identified protein. (g) Heat-map to the differential analysis of proteins identified in GEL and $CaSO_4$ samples.

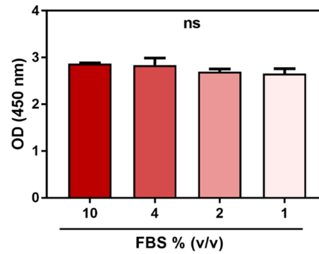


Figure 6. Effect of serum concentration of the culture medium on the metabolic activity of hBM-MSCs. The cells were cultured with the culture media supplemented with different ratios of fetal bovine serum for 24 h before metabolic activity was assessed. The metabolic activity of the cells was determined by Cell Counting Kit-8 assay following manufacturer's guidelines.

On the other hand, cells were cultured with a commercial serum-free medium, specially formulated for the growth and expansion of hMSCs that does not contain any animal component, during the whole culture period (StemPro® MSC XenoFree, Thermofisher) (XenoF Group). Concerning the exploited mass spectrometer, it is an innovative equipment designed for ultra-high sensitivity proteomics, which through the combination of the exclusive parallel acquisition serial fragmentation (PASEF) method and trapped ion mobility spectrometry (TIMS) technology improves notably the performance of identification and label-free quantitation (57).

Both the qualitative and quantitative analyses of the raw MS files were performed with the PEAKS software. Regarding the qualitative analysis, proteins identified with at least two unique peptides were considered to assess the efficiency of the protein extraction and the evaluation of their origin.

As can be seen in the Figure 5D, the total number of proteins identified in each sample increased substantially, thus several thousands of proteins were identified in this attempt. Regarding the distribution of these proteins according to the organism of origin, about 80% of the identified proteins in each sample correspond to human. However, the amount of bovine proteins was surprisingly similar in all groups, without remarkable differences between the samples cultured with serum containing medium and those samples grown with serum-free conditions. Besides, many of these proteins identified as bovine in the XenoF group samples presented high number of unique peptides for the bovine form, which are not present in the human form. For example, between 85 and 90% of the identified peptides were unique for the bovine form in the case of serum albumin (ALBU_BOVIN) and serotransferrin (TRFE_BOVIN) (Figure 5E), demonstrating the presence of bovine proteins in the XenoF samples. Therefore, we concluded that the origin of the detected background came predominantly from the gelatin scaffold itself and not from the culture medium. It must be taken into account that the scaffolds were composed of gelatin from bovine skin, and although

purification processes were carried out after its extraction from collagen, the presence of impurities might be plausible.

Nonetheless, considering the high number of proteins identified in each sample, the ratio of bovine proteins was not restrictive to proceed with the quantitative analysis of the samples. The quantitative analysis was carried out using the PEAKSQ module within PEAKS, where the intensity of each identified protein is determined for each sample. In order to have an overview of the biological functions in which the most abundant proteins may be involved, the averages of the intensities were calculated and the 2000 most intense human proteins were selected for the Gene Ontology analysis through DAVID software. Human proteome was considered as the generic background and Table 1 shows the most significant processes enriched of each category (Biological Process, Cellular Component and Molecular Functions). Interestingly, it seems that the cell-cell adhesion function could be one of the most relevant processes associated with the analyzed proteins. These intercellular connections are involved in the integration of cells into tissues and the mechanotransduction of biophysical processes governing functions such as cell proliferation, gene expression regulation and cell differentiation is partially arranged through these elements (58).

Table 1. Biological functions enriched with the most abundant identified proteins analyzed by Gene Ontology.

CATEGORY	TERM	p value
Biological Process	GO:0098609~cell-cell adhesion	1,4,E-83
	GO:0006413~translational initiation	9,1,E-69
	GO:0006614~SRP-dependent cotranslational protein targeting to membrane	8,1,E-53
	GO:0000184~nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	2,2,E-45
	GO:0019083~viral transcription	8,0,E-41
Cellular Component	GO:0005913~cell-cell adherens junction	8,5,E-93
	GO:0005840~ribosome	2,1,E-29
	GO:0000502~proteasome complex	7,8,E-28
	GO:0022625~cytosolic large ribosomal subunit	8,8,E-25
	GO:0022627~cytosolic small ribosomal subunit	5,3,E-20
Molecular Functions	GO:0098641~cadherin binding involved in cell-cell adhesion	2,0,E-94
	GO:0003735~structural constituent of ribosome	8,4,E-19
	GO:0003743~translation initiation factor activity	2,9,E-15
	GO:0003924~GTPase activity	5,3,E-15
	GO:0005525~GTP binding	1,6,E-14

Additionally, the intensities assigned to each protein and sample were analyzed by Perseus software to compare the proteomic profiles of cells seeded on different scaffolds (59). Proteins and samples were grouped based on their similarity in the heat-map depicted in Figure 5F. Samples with similar proteomic profile in terms of abundances are displayed next to each other. According to these results, the scaffold in which the cells were seeded has more impact on the proteomic profile than the medium used for their *in vitro* culture. It was possible to clearly discern between the GEL and $CaSO_4$_7 groups, while the HA_7 samples did not present any differential pattern. To dig deeper into the differences between the $CaSO_4$ and GEL groups, a *t*-test with correction of *p* values by multiple testing correction (FDR<5%) was applied, without considering which was the culture medium used. Proteins were considered significantly different between the groups when the value of *q* was less than 0.05 and the ratio higher than 2 in either direction. Nine hundred and eighty nine proteins satisfied these criteria, considering them significantly different between groups (Figure 5G). In this way, the analysis of these proteins by Gene Ontology or Ingenuity Pathway Analysis (IPA) could allow us to heighten the knowledge about modified functions in each of the groups and conclude how the scaffold composition can affect the hBM-MSCs behavior.

3.8 Bone regeneration in osteoporotic mice bone defect

The potential of gelatin/bioceramic composite scaffold as osteogenic growth factor delivery carrier promoting bone healing was assessed with osteoporotic mice calvaria defect model. The animals were divided into 5 experimental groups and the evaluation of the repair was performed 8 weeks after the induction of the critical-sized bone defect. The defects were empty in the control group and the developed 3D composite blank scaffolds or scaffolds loaded with BMP-2 were implanted in the rest of animals.

Although many researches have been completed around the development of carriers for osteoinductive growth factors, most of these approaches have been evaluated in healthy animals defect models. However, disparate results have been achieved regarding the bone repair with same formulation loaded with BMP-2 in healthy and osteoporotic rats. These findings suggest the necessity to assess the bone regeneration potential of developed systems in specific animal models (60). The combination of ovariectomy of female rodents and continuous administration of corticosteroids during 4 months has been previously validated as an osteoporosis model induction, showing altered structural characteristics of calvaria bone (61). In fact, the healing process of flat bones such as calvaria, is negatively influenced by osteoporosis disease. The delay on bone repair associated with osteoporosis is further complicated in critical-sized bone defects due to diminish self-healing ability of the diseased bones.

The histological images of the harvested defect areas revealed regenerated new bone tissue and bone microarchitecture in which developed 3D scaffolds were implanted (Figure 7A). Nevertheless, the repair response was limited in the control group (empty defect) and in the

groups with blank scaffolds without growth factor loading. In these experimental groups, the presence of newly formed bone was restricted to the margins of the defect and connective tissue that sometimes invaded the defect. In contrast, the functionalization of these scaffolds with BMP-2 growth factor allowed the increase of the repair response in comparison with the control group and the blank scaffolds. In these cases, around the half of the defect surface was occupied by newly formed bone.

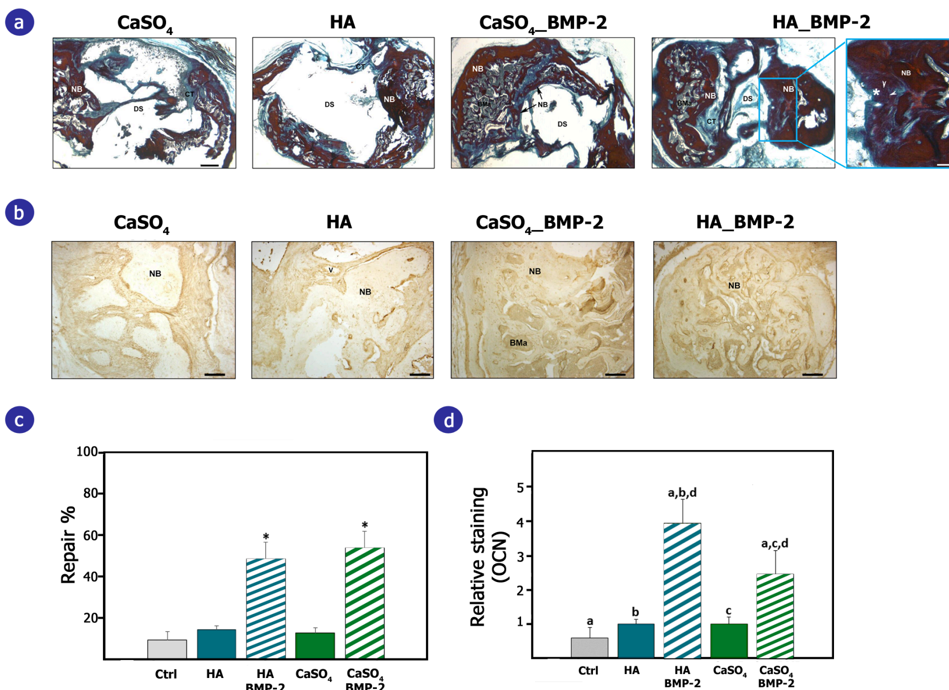


Figure 7. Bone regeneration assessment on osteoporotic mice calvaria defects 8 weeks after implantation of developed 3D scaffolds. (a) Representative VOF staining images in horizontal section showing the defect site in the different experimental groups. The region marked with the blue box stands out in the HA_BMP-2 group, which is shown at high magnification, shows an early mineralization zone defined as extracellular matrix of conjunctive-bone transition stained red (*) in which groups of osteocytes-like cells (arrowheads) are detected. Scale bars: Panoramic images = 500 µm. Detail image = 50 µm. (b) Representative images in horizontal section showing OCN immunoreactive staining in the defect site. Scale bar = 100 µm. (c) Quantitative analysis of histomorphometry showing the percentage of repair. (d) Relative OCN staining in arbitrary units in the different experimental groups. The same letters on different histograms indicates significant differences between these groups. BMa: Bone marrow, CT: Connective tissue, DS: Defect site, NB: Newly formed bone, v: Blood vessel. Statistical significance: $p<0.001$

Interestingly, the new bone tissue observed with the implantation of functionalized gelatin/bioceramic composite scaffolds showed mineralization levels comparable to normal animals. However, a more compact structure was detected with the implantation of HA_BMP-2 scaffolds, and several regions revealed early mineralization of an extracellular matrix of connective-bone transition (Figure 7A, indicated with *). Promisingly, in these areas groups of osteocytes-like cells also were observed (Figure 7A, indicated with arrowheads).

The quantitative histomorphometric analysis corroborated these observation and the repair percentages of control, $CaSO_4$ and HA groups were 9, 12.6 and 14%, respectively. Although a trend of improvement was detected with the implantation of blank scaffolds, these differences were not statistically significant. Conversely, the inclusion of the therapeutic factor BMP-2 in the developed systems improved notoriously the regeneration capability. Thus, the repair response with HA_BMP-2 and $CaSO_4$_BMP-2 were 48.4% and 53.5% respectively, without significant differences between them (Figure 7C).

To further explore the neotissue observed in the defect site 8 weeks after the 3D scaffolds implantation, OCN, a late marker of osteogenesis and mineralization, expression was analyzed (Figure 7B). The immunohistochemistry images revealed higher relative expression level of OCN in composite scaffolds loaded with BMP-2 groups in comparison to the control and blank scaffolds groups (Figure 7D). These results were in accordance with histological and histomorphometric data, suggesting the suitability of developed organic/inorganic composite scaffolds as osteogenic drug delivery carriers. Unlike those observed in the repair response from histological analysis, the OCN expression was significantly higher in HA_BMP-2 group compared to $CaSO_4$ scaffolds loaded with osteoinductive growth factor.

4. CONCLUSIONS

In this study, we have successfully engineered bioinspired organic/inorganic composite 3D scaffolds, integrating calcium sulfate and HA bioceramics into the enzymatically crosslinked gelatin networks. The reinforced systems showed higher pore size, increased compressive mechanical properties and good biocompatibility profile. In addition, hBM-MSCs exhibited efficient adhesion and proliferative capacities on the organic/inorganic composite scaffolds and osteogenic differentiation patterns were recorded in systems prepared with both bioceramics. The optimization of the experimental conditions and the potential proteomic analysis could elucidate the *in vivo* biological impact produced by the 3D scaffolds after their implantation on the host osteoprogenitor cells. Interestingly, these reinforced scaffolds were capable of promoting *in vitro* sustained release of BMP-2 after loading the therapeutic factor though soak-loading procedure, without the need of complex delivery systems. Thus, the osteoconductive gelatin/bioceramics composite functionalized with BMP-2 promoted bone regeneration in osteoporotic mice calvarial defect. Overall, the developed gelatin-based

composites may be useful as scaffolds for bone regeneration in osteoporotic defects, avoiding supraphysiological doses and reducing consequently the possible adverse effects.

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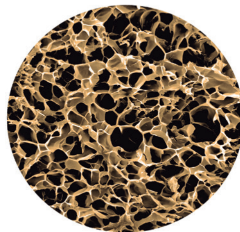
EXPERIMENTAL SECTION

CHAPTER 3



CHAPTER 3

Biphasic hydrogels integrating mineralized and anisotropic features for interfacial tissue engineering

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ABSTRACT

The innate graded structural and compositional profile of musculoskeletal tissues interfaces is disrupted and replaced by fibrotic tissue in the context of disease and degeneration. Tissue engineering strategies focused on the restoration of the transitional complexity found in those junctions present special relevance for regenerative medicine. Herein, we developed a gelatin-based multiphasic hydrogel system where sections with distinct composition and microstructure were integrated in a single unit. For that purpose, gelatin network was enzymatically crosslinked and hydroxyapatite (HA) particles and cellulose nanocrystals (CNC) were selected to adjust functionalities. Stiffer hydrogels were produced with the incorporation of mineralized particles and magnetic alignment of CNC resulted in anisotropic structure formation. The evaluation of the biological commitment with human adipose-derived stem cells (hASCs) towards tendon-to-bone interface, revealed an aligned cell growth and higher synthesis and deposition of tenascin (TNC) in the anisotropic phase, while the activity of the secreted alkaline phosphatase (ALP) and the expression of osteopontin (OPN) were induced in the mineralized phase. Overall, tendon and bone related features were assembled developing spatially differentiated regions. These results highlight the potential versatility offered by gelatin-transglutaminase enzyme tandem for the development of strategies that mimic the graded, composite and complex intersections of the connective tissues.

Keywords: anisotropic hydrogels, bone, gelatin, interfaces, tendon, transglutaminase.

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1. INTRODUCTION

The interfaces of the musculoskeletal system are graded connections between dissimilar connective tissues such as fibrous, calcified and cartilaginous materials. In these transitional regions, physical, chemical and biological features differ crosswise [1]. Articular cartilage, tendon/ligament enthesis or intervertebral disc are some examples of these transitional interfaces. These specialized complex regions present poor ability to promote the restorative processes upon injury or in diseases characterized by degeneration progress. In these cases, a fibrotic scar tissue with considerably different structure and composition from their native graded state is developed, often failing to fulfill their crucial mechanical functions [2].

In this scenario, interfacial tissue engineering, which involves interdisciplinary efforts for the development of substitutes that recapitulate the particular complexity present in these regions, is considered an attractive alternative to conventional reparative autograft or allograft transplantation [3]. The design, development and optimization of single unit constructs with spatially varied biochemical and structural cues are the main objective of these approaches. For that purpose, several processing techniques have emerged in the last years, and hydrogels systems in particular have been showing great progress in this filed [4]. These highly hydrated systems produced from a broad range of hydrophilic polymers simulate the extracellular matrix (ECM) of tissues [5]. Enzymatically crosslinkable hydrogels have gained increased interest because they can be formed under mild conditions and avoid some drawbacks related with other crosslinking strategies such as the potential cytotoxic effects induced by systems prepared with photo-initiators or the lack of stability associated to physically crosslinked hydrogels [6]. Peroxidases [7], transglutaminases [8] and tyrosinases [9], among others have recently been proposed for the production of hydrogels intended for use in regenerative medicine.

Although disparate polymers can be enzymatically crosslinked to produce hydrogels, gelatin owns unique properties that make it an exceptional biomaterial candidate. This natural origin, biodegradable and thermoresponsive polymer presents optimal physical-chemical and biological characteristics for biomedical applications that make it a widely studied material in tissue engineering approaches [10,11]. Numerous nano and micrometric fillers have been successfully combined with gelatin to produce composites that mimic musculoskeletal tissues. For example, biocompatible stiff osteoconductive gelatin/hydroxyapatite (HA) structures were developed by cryogelation technique for bone regeneration [12,13]. On the other hand, the mimicry of the anisotropic structure that characterizes tissues such as cartilage, tendons or ligaments is extremely important since their ordered ECM architecture and cellular organization play an essential role in the biomechanical and biological functions of these tissues [14]. Interestingly, different strategies using a range of polymers, including gelatin as the main matrix material, and manipulating the orientations and aggregation of

nanoparticles by external magnetic, electric or acoustic fields are being explored to create hydrogels with bioinspired ordered structures [15]. In this context, cellulose nanocrystals (CNC), rod-shaped nanoparticles extracted from the crystalline regions of the cellulose microfibrils with unique properties for tissue engineering applications such as excellent mechanical behavior, biocompatibility and sensibility to relative orientation under magnetic field [16], have received particular attention. Recently, it has been shown that is possible to manipulate CNC orientation within a hydrogel matrix using moderate magnetic fields despite their diamagnetic character [17]. Moreover, we have developed injectable anisotropic gelatin hydrogels loaded with CNC decorated with magnetic nanoparticles under exposure to uniform magnetic field of low strength [8]. Both studies have demonstrated that the anisotropic microstructure of the resulting hydrogels can induce the directional organization of seeded and encapsulated cells.

However, tissue engineering strategies that recapitulate the dissimilar composition, architecture and cellular organization found in the musculoskeletal tissue interfaces, in a single integrated construct have not been explored in depth. The development of such graded multiphasic hydrogels that allow cell encapsulation seems particularly challenging. In fact, current technologies to fabricate the pre-formed scaffolds often involve too harsh conditions for cells encapsulation and the assessment of *in vitro* biological performance is limited to cell seeding on the prefabricated scaffolds [18]. For instance, extreme pressure, non-physiological salt concentration and the use of organic solvents are some of those situations. Promisingly, the development of hydrogels under suitable conditions for three-dimensional (3D) cell culture provide more realistic biochemical and biomechanical microenvironments [19].

Here, we propose a novel methodology to develop a hierarchically structured hydrogel where mineralized and anisotropic phases are gradually integrated in a single construct. The natural origin polymer, gelatin, was enzymatically crosslinked with microbial transglutaminase, thus allowing the integration of the sections due to the remaining free reactive groups of gelatin and the enzyme catalytic activity. For the generation of 3D anisotropy within the hydrogel, incorporated CNC were magnetically aligned under the exposure to relatively low magnetic field strength (400 mT), whereas HA particles were added to provide osteoinductive properties to the system thus mimicking the composition and structure organization found in the transition zone of several musculoskeletal tissue interfaces. The morphological and mechanical properties of the composite hydrogels were thoroughly evaluated. Their biological performance was assessed by the encapsulation of human adipose-derived stem cells (hASCs) into the systems (mono or biphasic), and the alignment of the cell growth and the expression of ECM proteins were determined.

2. EXPERIMENTAL SECTION

2.1 Materials

Gelatin from porcine skin (Type A, gel strength 300), microcrystalline cellulose (MCC, Avicel), sulfuric acid 95-97%, phosphate buffered saline (PBS), bovine serum albumin, dialysis tubing cellulose membrane (MWCO 12-14 kDa), p-nitrophenyl phosphate (pNPP) and phalloidin tetramethylrhodamine B isothiocyanate (phalloidin-TRITC) were purchased from Sigma-Aldrich, Portugal. Microbial transglutaminase (mTG) was obtained from Ajinomoto, Japan. Hydroxyapatite (HA) microparticles were purchased from Fluidinova, Portugal. Neutral buffered formalin 10% (v/v) and Triton X-100 were obtained from ThermoFisher Scientific. Minimum essential medium alpha (α-EMEM), antibiotic/antimycotic (A/A) solution, fetal bovine serum (FBS) and TrypLE Express with phenol red were purchased from Life Technologies. Normal horse serum 2.5% was obtained from Vector Laboratories; 4,6-diamidino-2-phenyindole dilactate (DAPI) from Biotium, USA; and silicone squared molds (12 well chamber removable) from Ibidi, Germany. Alexafluor 488 donkey anti-rabbit and anti-mouse were purchased from Invitrogen.

2.2 Synthesis of cellulose nanocrystals (CNC)

The synthesis of CNC was achieved hydrolyzing MCC with sulfuric acid according to the protocol previously described by Bondenson *et al.*, with minor adaptations [20]. In brief, MCC/water suspension was prepared by mixing 42 g of MCC powder with 189 mL of deionized water and mechanically stirred at 500 rpm during 10 minutes, immersed in an ice bath to avoid overheating. Next, 188.3 mL of concentrated sulfuric acid 96% was added dropwise up to a final concentration of 64% w/w. The acid hydrolysis was performed heating the suspension to 44 ˚C and maintaining the vigorous stirring at 500 rpm for 2 hours. After that, the reaction was quenched by diluting the suspension with distilled cold water (5 times the initial volume) and the solution was left to decant at 4 ˚C for 2 hours. After discarding the acidic supernatant, the remaining suspension was centrifuged for 10 minutes, at 9000 rpm and 5 ˚C. The supernatant resulting after the centrifugation process was discarded and replaced by ultrapure water. The suspension was centrifuged until the supernatant became turbid. The resulting final suspension containing CNC was widely dialyzed against deionized water, using cellulose membranes (MWCO: 12-14 kDa) until neutral pH was reached. Consequently, the slurry content was poured into beakers and three cycles of 10 minutes sonication (VCX-130PB-220, Sonics) was applied to completely disperse the aggregates of nanocrystals. The ultrasound probe was set at 60% of amplitude output and an ice cooling bath was used to avoid overheating during the process. Finally, the cloudy suspension was centrifuged for 10 minutes at 9000 rpm and 5 ˚C to remove big particles and the final supernatant containing CNC was stored at 4 ˚C until further use.

Atomic force microscopy (AFM Dimension Icon, Bruker, USA) was used to characterize the produced CNC. One drop of 0.0015% w/v CNC suspension in ultrapure water was placed on a freshly cleaved mica disk, that after removing the excess liquid, was dried overnight. The AFM was used in PeakForce Tapping (ScanAsyst) in air mode with a MultiMode AFM connected to a NanoScope V controller (Veeco, USA). The scans were collected using a silicon nitride AFM cantilever (ScanAsyst-Air, Bruker) with a spring constant of 0.4 $N.m^{-1}$ and frequency of 70 kHz.

2.3 Development of enzymatically crosslinked hydrogels

Enzymatically crosslinked gelatin hydrogels were prepared using mTG enzyme. Nanocomposite hydrogels and mineralized hydrogels were developed by incorporating CNC or HA microparticles in the formulation, respectively. Gelatin from porcine skin was dissolved in 0.1X PBS solution at 10 % (w/v) under constant stirring at 60 ˚C for 2 hours and then cooled to 37 ˚C. The mTG solution was prepared at RT, dissolving the enzyme powder (100 U/g) at a concentration of 20% (w/v) in PBS. CNC suspension at different concentration (1 and 2 % (w/v)) was dissolved in ultrapure water, pH neutralized with 0.2 M NaOH and dispersed with ultrasonic processor (40% Amplitude output, 20 seconds, 3 cycles) prior to mixing with the polymer solution. Gelatin solution was mixed in a 1:1 (v/v) ratio with the CNC suspension and the required amount of mTG solution was added to obtain 10 U/g gelatin enzymatic activity. To prepare the final isotropic hydrogels, the resulting solutions were thoroughly mixed by pipetting up and down, dispensed into silicone-squared molds (6x6x4 mm) and kept at 37 ˚C in humid atmosphere for 1 hour to allow crosslinking. Instead, for the preparation of anisotropic hydrogels the molds containing the hydrogels solutions were kept under the influence of uniform magnetic fields in a custom-made magnetic system, consisting of two neodymium permanent magnets N52. Two different magnetic force levels were employed by adjusting the distance between the magnets, resulting in magnetic field strength of 200 mT and 400 mT. A gaussmeter (Hirst Magnetic Instruments) was used to determine the strength of the applied magnetic field. For the preparation of mineralized hydrogels, HA micrometric sized spherical particles (Particle size = 5.0±1.0 μm, Fluidinova, Portugal) were incorporated into the gelatin solution. Both the gelatin and HA particles were dissolved at the same time, using the conditions described above. Similarly, the necessary volume of enzyme was added to the mixture, and the crosslinking process was completed at 37 ˚C for 1 hour. Moreover, biphasic hydrogels with differentiated sections were developed by casting the pre-solution of a new hydrogel on the top of the crosslinked hydrogel. Thus, mineralized isotropic phase and nanocomposite anisotropic phase were overlaped. The composition and the preparation conditions of each hydrogel are summarized in the table of Figure 1D.

2.4 Characterization of developed hydrogels

2.4.1 Scanning electron microscopy (SEM)

The microstructural morphology and organization of nanocomposite and mineralized hydrogels was analyzed by high-resolution SEM (JSM-6010LV, JEOL, Japan). The samples were freeze-dried first, cooling and fractured in liquid nitrogen then, and sputter-coated with platinum after (Cressington). The image acquisition was performed with an acceleration voltage of 10 kV. ImageJ software was used for the analysis of the images. SEM images were converted to binary and erode or dialate functions were applied then. These adjusted files were used for the determination of shape description parameters via the analyze particles function. At least 50 pores per formulation were assessed. For the analysis of the organization of the pores, directionality determination was performed using the Fourier components method.

2.4.2 Mechanical characterization

The compressive mechanical behavior of the developed hydrogels were analyzed using an universal mechanical testing equipment (5543K2942, 5543, Instron) equipped with a 1 kN load cell, under a compression rate of 1 mm/min. The samples were prepared in molds with a uniform cylindrical shape (6x6x5 mm). Prior to the test, the size of the hydrogels was measured using a digital caliper. The Young´s compressive modulus was determined from the slope of the stress-strain curve in the 10-25% strain linear region. Same experimental settings were applied to evaluate at least five different samples per each composition.

2.4.3 Rheological analysis

The rheological properties of the nanocomposite hydrogels were measured using a rheometer AR1000 (TA instruments, New Castle, USA) and a test geometry of 40 mm diameter plate was used. 1 mL of the solutions was directly dispensed while in liquid state on the bottom Peltier plate, previously heated at 37 ˚C. The gap was adjusted to 0.5 mm and a thin film of paraffin oil was used to avoid the water evaporation from the samples. Time-sweep tests were conducted at a frequency of 0.5 Hz and shear strain of 0.5%, monitoring values of storage (G') and loss moduli (G") for 75 minutes. Isotropic nanocomposite cylindrical hydrogels were prepared for the analysis of bulk viscoelastic properties. For that, oscillatory frequency sweep tests (0.01 - 10 Hz) were carried out at 37 ˚C, applying an initial pre-adjusted normal force of 1 N and a constant strain amplitude of 0.5%. Three replications were performed for each formulation, representing the average of those values as the final result.

2.4.4 Polarized optical microscopy

Polarized optical microscopy was used to evaluate the anisotropic hydrogel network and the time required to achieve the alignment of the CNC under magnetic field. Thin hydrogels were produced by deposition of 100 μL of the hydrogel precursors in the molds mentioned above. The formulations were physically crosslinked at each time point by cooling the system

with an ice bath. Images were acquired using an optical microscope equipped with a digital camera. Samples were placed between crossed polarizers and 10x magnification was used for all images.

2.4.5 Micro-computed tomography (μ-CT)

The biphasic hydrogels were assessed by high-resolution μ-CT analysis (Skyscan 1272, Skyscan, Belgium). Two dimensional projections with a pixel resolution of 4.96 μm were acquired over a rotation range of 360° and with a rotation step of 0.4°, by cone-beam acquisition after irradiating the specimens with penetrative X-rays using a source voltage of 60 kV and a current of 166 μA. The 3D image was reconstructed using the software NRecon (version: 1.7).

2.5 Biological performance of hydrogels

2.5.1 Cell culture

Human adipose tissue-derived stem cells (hASCs) were used to evaluate the biological behavior of the developed hydrogels. hASCs were isolated from lipoaspirate samples obtained from Hospital da Prelada (Porto, Portugal). All the procedures were approved by the Ethical Committee of the Hospital da Prelada and University of Minho. The hASCs isolation and stemness characterization were performed following a protocol described elsewhere [21]. Briefly, the tissue samples were thoroughly washed with PBS and digested with 0.05% collagenase Type I A (Sigma-Aldrich) in PBS for 60 min at 37 ˚C under gentle stirring. Then, cells were centrifuged and the cell pellet was resuspended in basal cell culture medium (α-MEM supplemented with 10% (v/v) FBS and 1% (v/v) A/A) and seeded in culture flasks. After 24 hours of incubation at 37 ˚C and 5% CO_2, the adherent cells were washed and their stemness was evaluated through flow cytometry for the expression of mesenchymal stem cell markers (CD45, CD105 and CD90), as shown previously [22]. For all experiments, hASCs were maintained in culture with basal medium and used at passage 3-6.

2.5.2 Cell encapsulation in 3D hydrogels

The hASCs cells were encapsulated in the 3D hydrogels. The gelatin 10% (w/v) solution prepared in 0.1X PBS was supplemented with 25 mM of sucrose, with the aim of getting a suitable environment for cellular growth, adjusting the osmotic pressure for it. This solution and the dissolution of mTG enzyme were filtered using 0.22 μm filters. On the other hand, the suspension of nanocrystals was first dispersed by ultrasonic processor (40% amplitude, 30 seconds, 3 cycles) and placed under UV radiation for 30 min after. The cells were resuspended in the gelatin solution to obtain 10^6 cells per mL of hydrogel. The cellular suspension in gelatin, the enzyme solution and the CNC suspension were mixed as described before and the volume necessary to achieve 1% A/A in the final formulation was added. The isotropic and anisotropic hydrogels were prepared as previously described by adding 100 μL of the mixture in the silicon squared molds mentioned above. The cell containing hydrogels were kept at

37 °C during 60 min for the crosslinking phase. After that, the hydrogels were removed from the molds and placed in 24 well plates. 1 mL of basal medium was added to each well, changing it every 2 days over the course of the cellular assays.

2.5.3 Cell organization evaluation

Cell nuclei and cytoskeleton actin filaments were stained and confocal laser scanning microscope (Leica TCS SP8, Mycrosystems, Wetzlar, Germany) was used to evaluate the cellular morphology and organization. At each time point, the hydrogels with encapsulated hASCs were fixed with neutral buffered formalin 10% (v/v) at RT for 30 min. After washing the samples two times with PBS, a solution of Triton X-100 at 0.2% (v/v) in PBS containing DAPI (1:500, v/v) and phalloidin-TRITC (1:200, v/v) was used for the staining, maintaining 1 hour protected from light under gentle shaking (130 rpm). A final washing step with PBS was conducted to eliminate the excess dye. Images from the samples were acquired through confocal microscopy. Nuclei aspect ratio was evaluated measuring at least 50 nuclei for each condition using Image J software. The nuclei aspect ratio was estimated by dividing the length by the width, achieving ratios > 1 for elongated nuclei. For cytoskeleton organization evaluation, directionality plugin with Fourier components method from Image J software was applied to the images corresponding to actin filaments. The histograms were fit to the Gaussian distribution function using Origin software. As a result, the full width at half medium (FWHM) was determined for each group.

2.5.4 Immunofluorescence staining for bone and tendon-related markers

Immunostaining for specific osteogenic and tenogenic markers was conducted after 6 and 21 days of culture. Before the staining, hydrogels were washed with PBS and fixed in formalin 10% (v/v). For cell permeabilization Triton X-100 at 0.1% (v/v) in PBS was used for 20 minutes under gentle agitation. After rinsing the samples three times with PBS, normal horse serum 2.5% was added to block unspecific reactions and maintained for 90 minutes at RT. Then, hydrogels were incubated with primary antibodies against osteopontin (OPN) (rabbit anti-osteopontin antibody, 1:1000, Abcam ab8448) and tenascin (TNC) (mouse anti-tenascin-C antibody, MAI-26779, ThermoFisher Scientific, 1:3000). The samples were incubated overnight at 4 °C under gentle shaking. After removing the antibody solution, a washing step with H_2O_2 at 0.3% (v/v) for 15 minutes was carried out. Thereafter, the samples were incubated with the corresponding secondary antibody labeled with AlexaFluor 488 (1:200), for 2 hours at RT protected from light under mild agitation. All the antibodies were prepared in BSA 0.1% (w/v) in PBS. Finally, once the samples were washed with PBS, nuclei and cytoskeleton were stained with DAPI (1:500 in PBS) and phalloidin-TRITC (1:200 in PBS), respectively, at RT for 1 hour. After a final washing, the samples were kept in PBS at 4 °C until the imaging. The immunostained hydrogels were analyzed with the confocal microscope. The maximum projections for Z stacks confocal images were obtained and

the evaluation of specific signal was normalized per nuclei area. The pixels corresponding to background signal were discarded applying a threshold, and the area of immunolabeled pixels was divided by the area corresponding to blue nuclei pixels. In the case of TNC staining, the directionality analysis was also completed following the same protocol described for the cytoskeleton organization evaluation.

2.5.5 Alkaline phosphatase (ALP) activity determination

The secretory form of ALP from hASCs was monitored during 3 weeks. The activity was evaluated by determining the hydrolysis of pNPP by ALP at pH 9.3. A standard curve with calf intestinal ALP was prepared and 100 µL of pNPP at 0.2% (w/v) was added to each sample. The reaction was stopped with 50 µL of NaOH 3M and absorbance measured at 405 nm.

2.6 Data analysis and statistics

The statistical analysis of the data was completed using GraphPad PRISM (7.0) software. For normally distributed data, Student´s *t*-test or one-way ANOVA were applied for differences between two groups or multiple comparisons, respectively. Tukey *post-hoc* test was applied for multiple comparisons. For non-normally distributed data, Mann-Whitney nonparametric analysis or Kruskal-Wallis test with Dunn´s multiple comparisons test were applied. In all cases, p values <0.05 were considered as significant, represented by symbols described in the graphs. Data are presented as mean ± standard deviation.

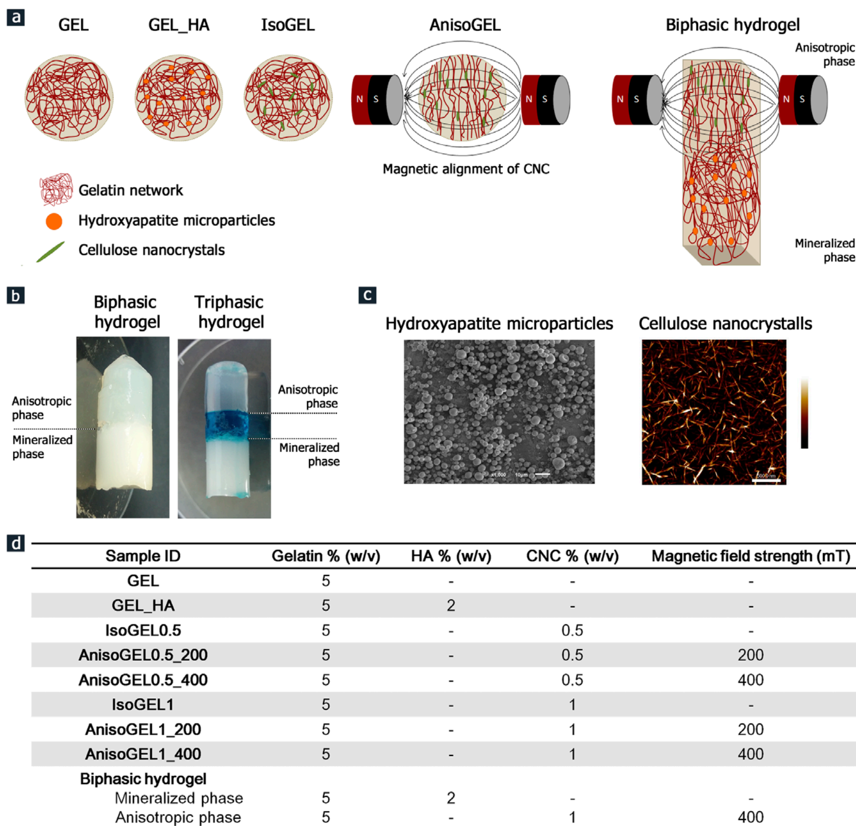
3. RESULTS AND DISCUSSION

3.1 Development of enzymatically crosslinked hydrogels

Natural origin polymeric hydrogels were designed, using gelatin as the main component. The gelatin network was enzymatically crosslinked by mTG enzyme and specific micro and nanometric particulate fillers were incorporated into the constructs, in order to obtain hydrogels with specific tissue mimetic characteristics (Figure 1A). In this regard, HA spherical 5 µm microparticles were selected to provide osteoinductive features to the formulation (Figure 1C) [23,24]. HA is the main inorganic component of the mineral phase of native bone tissues. Furthermore, it is well established the capability of this material to support osteogenic differentiation of mesenchymal stem cells from different sources, such as adipose tissue [25] or bone-marrow [26,27]. On the other hand, rod-shaped CNC were incorporated into the formulations to act as both reinforcement nanomaterial and as a guide for the development of anisotropy under magnetic stimulation (Figure 1C). These nanoparticles are broadly used in tissue engineering approaches to achieve added functionalities to hydrogels systems, such as electrical or magnetic responsiveness [28].

The enzyme applied for the fabrication of these systems catalyzes the reaction between ε-amino group of lysine and γ-carboxamide group of glutamyl residues, giving as a result

stable intra and intermolecular isopeptidic bonds in physiological conditions [29]. This enzymatic strategy of getting covalently crosslinked gelatin hydrogels has been previously proposed by other authors to design biocompatible *in situ* gelling injectable formulations without remarkable immunogenic or cytotoxic aspects [30]. In addition, this enthralling approach presents an exceptional versatility to combine diverse compositional hydrogels. In this work, unique structures composed of differentiated phases (mineralized phase vs anisotropic phase) with well-integrated interfaces have been successfully developed. Biphasic or even triphasic systems (Figure 1B) were produced by stacking a new gelatin solution on the top of previously crosslinked hydrogel. The catalytic activity remaining on the surface of the preformed structure allows the integration of the new gelatin network.



Sample ID	Gelatin % (w/v)	HA % (w/v)	CNC % (w/v)	Magnetic field strength (mT)
GEL	5	-	-	-
GEL_HA	5	2	-	-
IsoGEL0.5	5	-	0.5	-
AnisoGEL0.5_200	5	-	0.5	200
AnisoGEL0.5_400	5	-	0.5	400
IsoGEL1	5	-	1	-
AnisoGEL1_200	5	-	1	200
AnisoGEL1_400	5	-	1	400
Biphasic hydrogel				
Mineralized phase	5	2	-	-
Anisotropic phase	5	-	1	400

Figure 1. Schematic representation of developed gelatin-based hydrogels. (A) Enzymatically crosslinked gelatin networks reinforced with HA or CNC particles, achieving anisotropy under magnetic field exposure. (B) Digital images of produced biphasic and triphasic hydrogels with well-differentiated phases. Blue dye was used to better differentiate the transition between phases. (C) SEM image of spherical HA microparticles and AFM image of CNC. (D) Summary table of the composition and preparation conditions of the developed hydrogels. A sample ID given to each system is applied throughout the entire manuscript.

3.2 Characterization of gelatin nanocomposite constructs

Morphological characterization was completed by microstructure analysis of the hydrogels representative SEM images. Freeze-dried isotropic and anisotropic hydrogels with 0.5% and 1% (w/v) CNC were cross-sectioned in order to evaluate the internal organization. As shown in the images depicted in Figure 2A, porous structures were obtained in all cases. Concerning this aspect, randomly oriented porous without any organization were visualized in isotropic samples prepared in the absence of magnetic field. However, more organized arrangement was observed in those hydrogels that were exposed to magnetic alignment during the cross-linking phase. These structural differences were proved by directionality analysis of the images (Figure 2B). The structural alignment observed in the anisotropic systems, resulted in a pointed frequency peak at a particular angle of orientation, without any remarkable difference between both concentrations. Conversely, multimodal flat distribution was obtained for both isotropic hydrogels. Regarding the morphology of the pores, lower data scattering of the aspect ratio was achieved for anisotropic systems (Figure 2C), demonstrating higher shape regularity in those samples. Furthermore, regarding the size of these pores, larger pores were formed with structural alignment, which may be due to the spatial reorganization of the gelatin strands. Nonetheless, higher concentration on CNC resulted in smaller pores (Figure 2C). However, the pore size of all these formulations (6-22 µm) falls within the range to allow the diffusion of oxygen, nutrients and metabolic products to cells [31].

To further evaluate the anisotropy of the proposed systems upon exposure of 200 and 400 mT magnetic fields, polarized optical microscopy was used. Continuous multi-chromatic transmission images were observed in the case of gelatin nanocomposite gels produced without external magnetic exposure (IsoGEL0.5 and IsoGEL1). This result shows the development of multi-domain structures with heterogeneous birefringence due to the random organization. In contrast, a chromatic transition from multichromatic to monochromatic is displayed in few minutes when the systems are exposed to a low uniform magnetic field (200 mT) (Figure 2D). Monochromatic images were visualized after 15 minutes of magnetic exposure for the hydrogels containing both concentrations of CNC tested. This result reflects the existence of a mono-domain structure that presents homogenous birefringence covering the entire region. However, this effect was achieved faster with the hydrogels containing 1% (w/v) CNC since images of a single color were observed after 10 minutes. Moreover, as it can be seen in the images depicted in Figure 3, by increasing the applied magnetic field strength to 400 mT, faster CNC alignment is achieved, getting mono-domain structures at 10 minutes in both cases. These results are in accordance with previously conducted studies on the analysis of the magnetic alignment process of CNC suspensions, where fast initial ordering within minutes in response to increasing magnetic field strengths was observed [32]. Similarly, Omidinia-Anarkoli A *et al.* concluded that the orientation time of magneto-responsive

poly(lactide-co-glicolide) microfibers containing superparamagnetic iron oxide nanoparticles decreases with increasing the magnetic field strength [33].

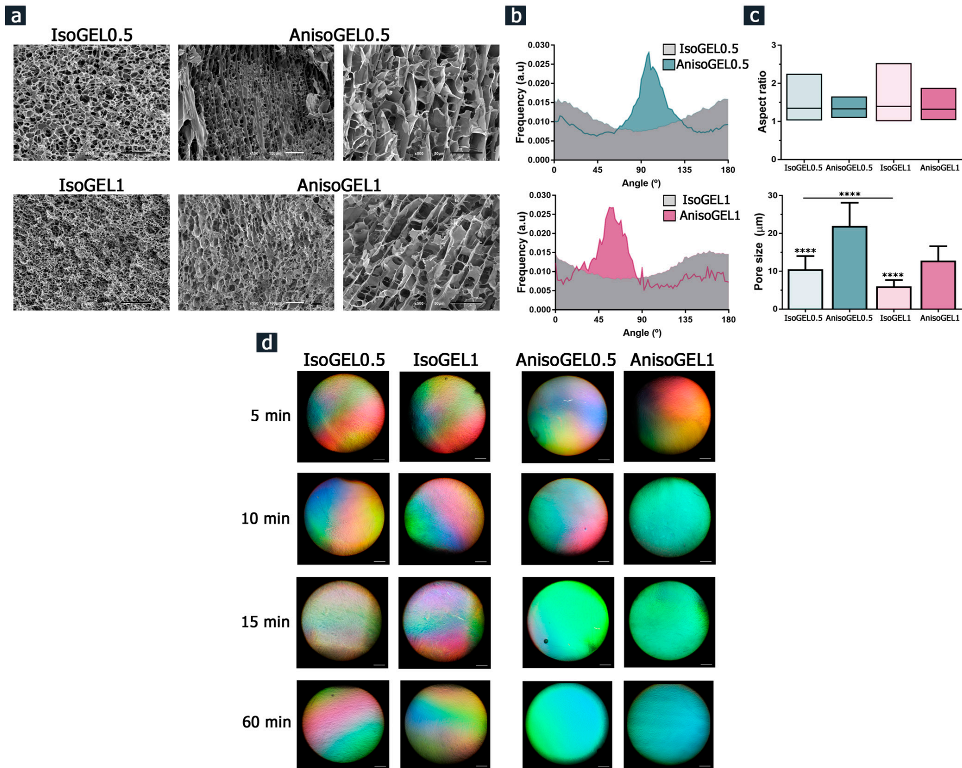


Figure 2. Morphological characterization and optical properties assessment of CNC containing hydrogels. (A) Scanning electron micrographs of the freeze-dried hydrogels (scale bar = 50 µm). (B) Structural directionality evaluation of isotropic and anisotropic hydrogels. (C) Analysis of descriptive parameters of the pores; the aspect ratio and pore size. Statistical differences: **** $p<0.0001$. (D) Polarized optical microscopy images taken during the crosslinking phase with or without 200 mT magnetic field exposure (scale bar = 500 µm).

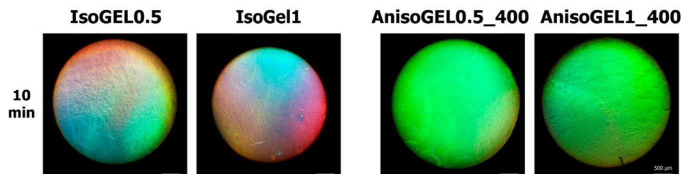


Figure 3. Polarized optical microscopy images taken after 10 min with or without 400 mT magnetic field exposure. Hydrogels containing 0.5 and 1 % (w/v) cellulose nanocrystals were tested in this assay. Scale bar = 500 µm.

To study the impact of CNC incorporation and alignment on the rheological and mechanical properties of the developed hydrogels, we performed time-sweep tests to determine the gelling point, frequency-sweep to assess the bulk viscoelastic properties and compression tests to evaluate the reinforcing effect achieved. The incorporation of the CNC within the gelatin network enzymatically crosslinked with the mTG enzyme proved to have no influence on the gelation time. In all cases, the formulations were fully crosslinked and the 3D systems were structured between 17 and 20 minutes (Figure 4A), thus, showing that the gelling time is enough to allow the alignment of CNC (Figure 2D). This time-range is similar to other *in situ* gelling injectable formulations proposed for tissue engineering applications [34,35].

With respect to isotropic hydrogels bulk viscoelastic properties (Figure 4B), data exhibited an increase in the elastic modulus (G') value proportional to the concentration of nanoparticles used. Hence, gelatin hydrogel without reinforcing nanofiller showed G' of 524 ± 12 Pa, while up to 4-fold stiffer isotropic hydrogel were developed with 1% (w/v) CNC incorporation (2450 ± 209 Pa). These results demonstrated the convenience of these nanoparticles as reinforcing elements, due to the good dispersion within the matrix.

Finally, this reinforcing effect, together with the alignment of the CNC under exposure to different strength magnetic fields, was further confirmed by the analysis of compressive properties. As can be seen in Figure 4C, the higher the proportion of CNC in the hydrogel, the stiffer structures were obtained. The hydrogel prepared with 0.5% (w/v) CNC in the absence of magnetic field showed Young modulus of 17.36 ± 4.12 kPa, while the formulation comprised of 1% (w/v) and produced under the same conditions presented compressive modulus of 27.31 ± 6.83 kPa. Besides, the exposure to a magnetic field during the gelation process increases compressive properties, confirming the presence of anisotropic microstructures in these hydrogels. Reasonably, the existence of strictly arranged planes of aligned rigid CNC perpendicularly oriented to the course of compression might enhance the resistance to compression of the hydrogel [36]. In fact, the highest Young Modulus was achieved by AnisoGEL1_400 sample (38.37 ± 4.85 kPa).

3.3 Biological performance of CNC-loaded hydrogels

hASCs were used to evaluate the biological performance of gelatin nanocomposite hydrogels. First, a preliminary study was completed to determine the biocompatibility of the enzymatically crosslinked nanocomposite systems. For that, the viability of cells encapsulated into the isotropic hydrogels was evaluated by live/dead staining. As can be seen in Figure 5, the cells cultured in the 3D systems for three days showed high viability, without significant differences between groups. These results demonstrate that the designed nanocomposite biomaterials are not cytotoxic and that they could be suitable for cellular growth support.

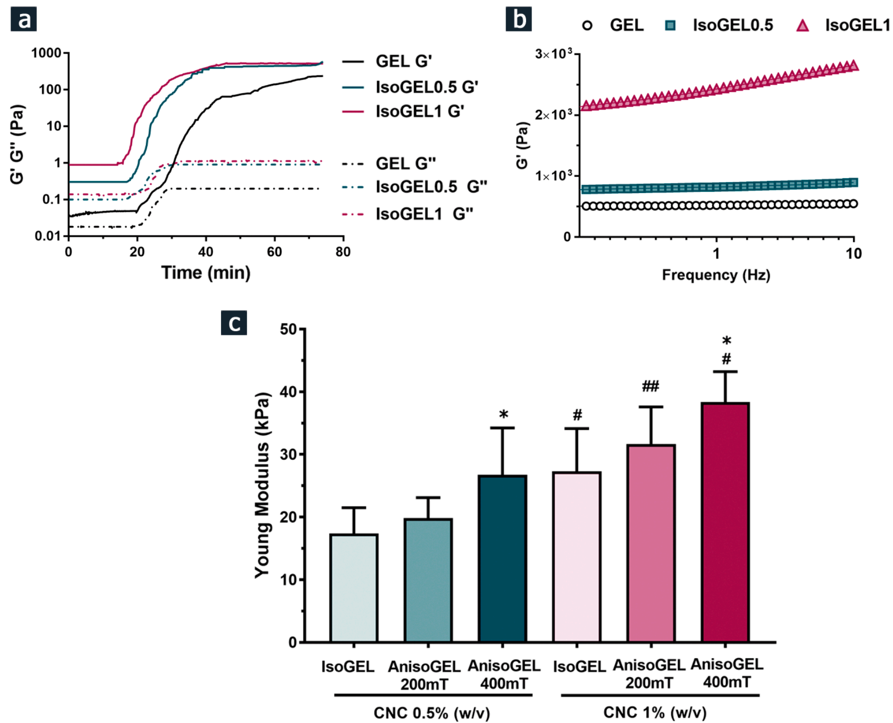


Figure 4. Mechanical characterization of CNC containing hydrogels. (A) Rheological time-sweep curves of isotropic hydrogels with 0, 0.5 and 1% (w/v) CNC. (B) Elastic modulus (G') from frequency-sweep of isotropic systems. (C) Young Modulus determined from strain-stress curves linear region under compression force. Statistical differences: * $p<0.05$ compared with isotropic hydrogel with the same concentration of CNC; # $p<0.05$ and ## $p<0.01$ compared with the hydrogels exposed under the same magnetic field.

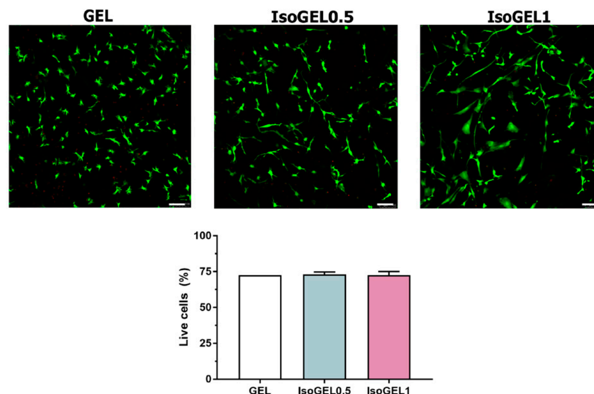


Figure 5. Confocal images of hASCs encapsulated in gelatin hydrogels with 0, 0.5 and 1 % (w/v) cellulose nanocrystals after 3 days of culture and the live/dead staining. Quantitative data from the images acquiered are shown. Scale bar = 100 μm.

Next, the effect of hydrogel alignment on cytoskeleton organization was determined. Constructs containing 0.5 and 1% (w/v) CNC were exposed to uniform magnetic fields of 200 and 400 mT during the crosslinking phase. Isotropic hydrogels containing the same amount of CNC but crosslinked in absence of external magnetic stimulus were considered as controls. The cellular orientation was observed under confocal microscopy after 3, 7, and 21 days of culture.

After 3 days of culture, hASCs encapsulated within the anisotropic hydrogels displayed a spindle-shape morphology and they were more spread than in control hydrogels (Figure 6A). Besides, it was possible to appreciate a certain orientation preference in the direction of these cells growth. Therefore, different frequency distribution profiles were captured from cytoskeleton directionality analysis. While multimodal flat distribution was shown by both isotropic hydrogels, unimodal narrow distribution tendency could be deduced from all anisotropic hydrogels histograms. These data were further confirmed from the Gaussian distribution fitting, since FWHM values of anisotropic hydrogels were significantly lower (FWHM ~ 40°) compared with the analogue isotropic samples (FWHM = 121.3 ± 34.65° for IsoGEL0.5 and 148.3 ± 44.81° for IsoGEL1). Indeed, the size, geometry or even the spatial organization of nanotopographical cues could have influence on stem cells adhesion, migration, proliferation, morphology and differentiation fate, essentially through coordinated mechanotransduction system. Several molecular pathways such as FAK/Src, Rhoa/ROCK or LINC complex are responsible for the transference of stimulus from ECM to the nucleus [37].

After 7 days of culture, the cell density was notoriously higher in all the formulations, which suggests a suitable cell proliferation thereof. hASCs encapsulated in gelatin-based nanocomposite hydrogels were overall fusiform and they were completely spread through the 3D environment (Figure 6B). In the case of cells embedded into isotropic systems, random organization without any preferential orientation was observed. Otherwise, in the case of anisotropic hydrogels, the cellular alignment observed after 3 days of culture was maintained also after 1 week. This alignment was confirmed by directionality analysis and sharp peaks at specific angles were noticed for anisotropic constructs, getting values of FWHM around 60°. Taking into account that no magnetic field was applied during the entire culture period, it is suggested that the cellular alignment observed at 7 days was an outcome of the intrinsic anisotropy of the hydrogels, as suggested in previous studies using anisotropic systems [8,38]. Regarding the influence of the magnetic field strength used to produce anisotropic hydrogels on cell organization, no significant differences were noticed between 200 or 400 mT exposed hydrogels. These results suggest that although the higher magnetic field used in this study leads to increased hydrogel physical anisotropy, it is not different enough to impact 3D cell organization. Therefore, considering the overall physic-chemical properties and the influence over cell organization, AnisoGEL1_400 formulation was selected for further cellular studies.

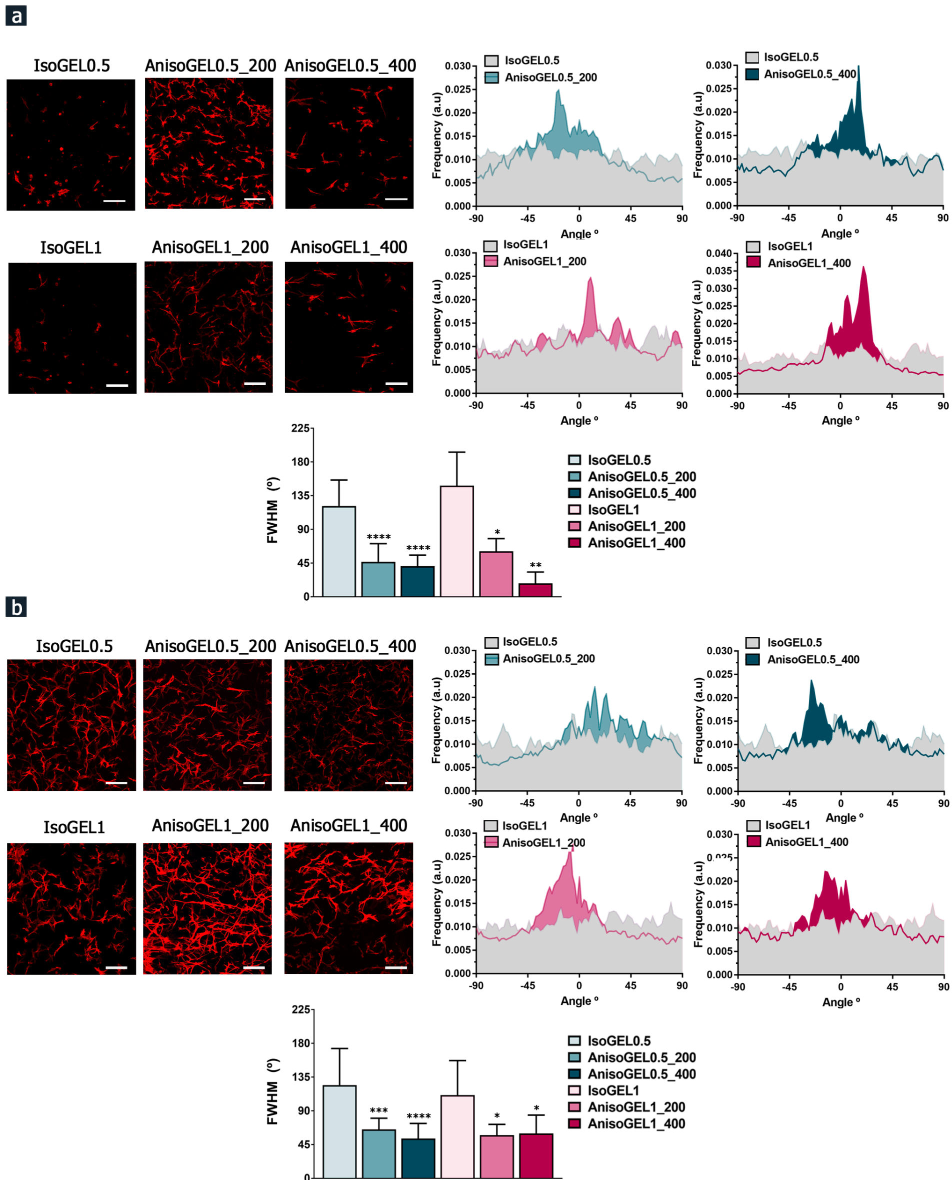


Figure 6. Evaluation of encapsulated hASCs cytoskeleton alignment in 3D hydrogels. Confocal fluorescence images of cellular cytoskeleton (F-actin, red) (scale bar = 200 μm), the directionality histograms of actin filaments distribution and the full width half medium (FWHM) of the histograms determined by Gaussian fitting of the curves after 3 days (A) and 1 week (B) of culture. Statistically significant differences between isotropic and anisotropic hydrogels with the same concentration of CNC are shown as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Selected formulations were maintained until 21 days of cell culture (IsoGEL1 and AnisoGEL1_400) and morphological and biochemical cues related to anisotropic tissues were evaluated then. Thus, nuclei aspect ratio, directionality of cell distribution and tendon-related ECM protein TNC secretion [39] were assessed by confocal immunofluorescence (Figure 7A). The nuclei of cells encapsulated within magnetically assisted platforms showed a tendency to elongate, obtaining higher values in nuclei aspect ratio (Figure 7B). Accordingly, the alignment of the cytoskeleton was much more pronounced for these samples, presenting a main orientation against the random distribution maintained of the isotropic systems (Figure 7C). Finally, the amount of TNC expression per nuclei was greater in the anisotropic hydrogel (Figure 7E) and the deposition of this glycoprotein was displayed in a much more fibrillar and organized way, being consistent with the cell alignment orientation (Figure 7D). This oriented structural arrangement of secreted TNC has been previously described. For example, controlled parallel spatial deposition of ECM proteins to aligned polycaprolactone/silk fibroin nanofibers from seeded dermal fibroblasts was concluded in rabbit Achilles tendon defect model [40].

All together, the data achieved with AnisoGEL1_400 hydrogel suggest its potential to simulate anisotropic environment found in some native tissues, such as tendon tissue.

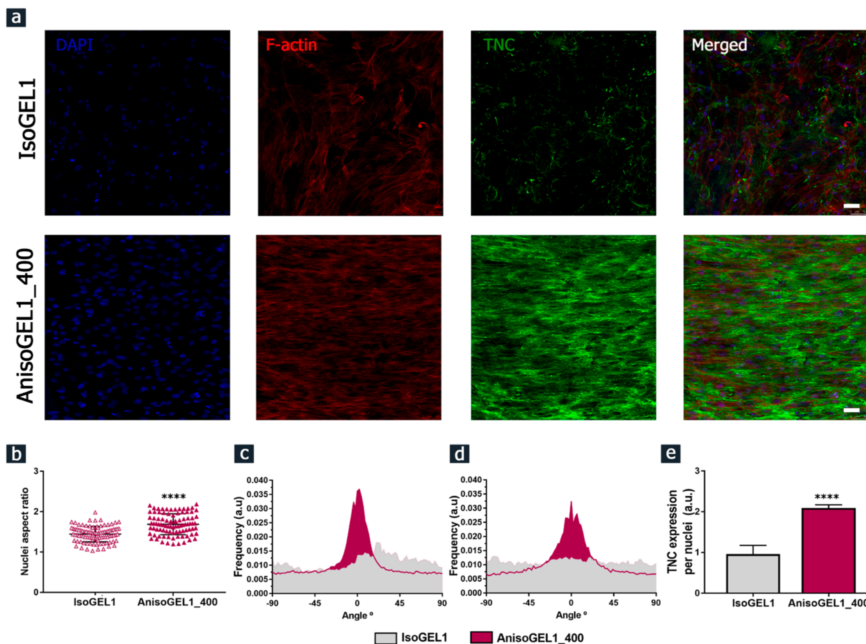


Figure 7. Tenascin (TNC) expression by hASCs encapsulated in nanocomposite 3D hydrogels after 21 days of culture. (A) Confocal images of immunolabeled samples against the TNC (green), cells nuclei (blue) and cytoskeleton (red) (Scale bar = 100 µm). (B) Nuclei aspect ratio of cells cultured in isotropic and anisotropic (400 mT) hydrogels reinforced with 1% (w/v) CNC. Evaluation of the cytoskeleton (C) and the TNC deposition (D) directionality by the encapsulated cells. Quantification of tendon-related marker expression normalized with nuclei area. Statistical significance: **** $p<0.0001$.

3.4 Mineralized hydrogels: characterization and evaluation of bone commitment by hASCs

HA particles were added into the enzymatically crosslinked gelatin hydrogels, in order to get mineralized hydrogels to better mimic the bone phase present in the graded interfaces of the musculoskeletal system. The osteoinductive microparticles were broadly distributed around the entire hydrogel network, well-dispersed without appreciable tendency to aggregate. The evaluation of the freeze-dried samples by SEM allowed assessing the disposition of the particles into the 3D system. These images are depicted in the Figure 8A, and it was confirmed that the mineralized particles were wrapped by the gelatin network. Therefore, constructs displaying porous microstructures with mineralized cues distributed throughout the system were developed.

Regarding the compressive properties of the mineralized hydrogels, significantly higher Young Modulus (43.28 ± 10.14 kPa) was achieved in comparison to control hydrogels without reinforcing material (Figure 8B). This reinforcing effect might be interesting to those constructs aimed at stiffer tissues applications. In fact, this value is in the same range of osteoid stiffness, the bone precursor crosslinked collagen secreted by osteoblasts [41].

For the biological performance evaluation of mineralized hydrogels, hASCs were encapsulated in 3D constructs and bone-related markers were analyzed, comparing the data with unloaded gelatin hydrogels. The activity of ALP secreted by the cells was used to determine the potential of the system to guide osteogenic differentiation of hASCs [26,42]. Greatly different profiles from the two tested samples were found (Figure 8C). Whereas the activity of ALP secreted by cells encapsulated within hydrogels composed only of gelatin was maintained at baseline levels during the 3 weeks of the study, the cells in the GEL_HA systems gradually increased the secreted ALP. Thus, the activity determined at the end point for GEL_HA was significantly higher than that corresponding to GEL hydrogel and the value obtained in the first week of the cellular experiment ($p<0.0001$). Additionally, the expression of OPN was evaluated after 6 and 21 days of culture by immunostaining. The representative fluorescence images and the correspondent quantitative analysis are shown in the Figure 8E and D. As expected, enhanced expression of this osteogenic marker was accomplished with mineralized hydrogels. OPN is a calcium-binding protein involved in the process of ECM calcification. The high levels of this marker are associated with the process of ECM mineralization that comes up during the late osteogenic differentiation of osteoprogenitor cells [43]. These results confirm the osteoinductive properties of this mineralized biomaterial, presenting the capability to promote osteogenic differentiation of hASCs cultured without osteogenic supplements.

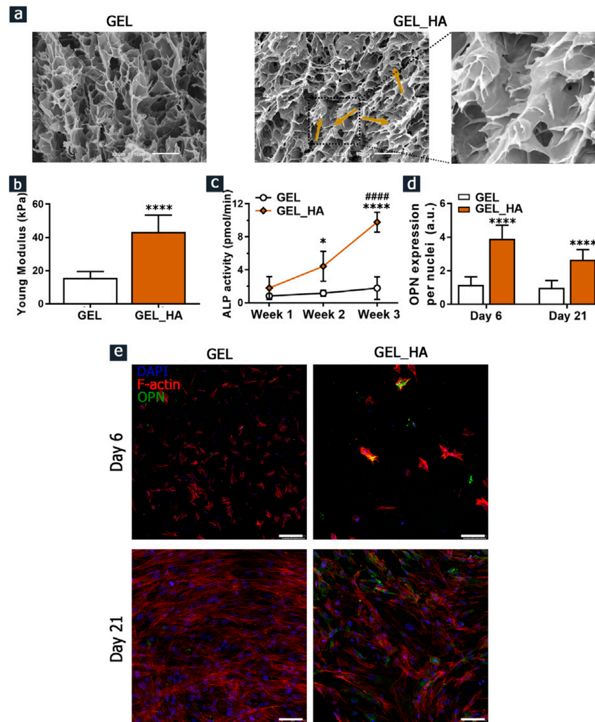


Figure 8. Physical and biological characterization of mineralized hydrogels. (A) Representative SEM images of freeze-dried gelatin hydrogels and hydroxyapatite (HA) containing systems. The arrows point the HA particles distributed around the structure (Scale bar =10 μm). (B) Young Modulus of the hydrogels obtained under compression tests. Statistical differences: $p<0.0001$. (C) Determination of alkaline phosphatase (ALP) activity secreted by hASCs after 1, 2 and 3 weeks of culture. Statistically significant differences between groups at each culture time are shown as * $p<0.05$ and **** $p<0.0001$. (D-E) Confocal images and quantification of the Osteopontin (OPN) expression (green) at 6 and 21 days of culture with nuclei and cytoskeleton stained in blue and red, respectively. Statistical differences: **** $p<0.0001$ (scale bar = 75 μm).

3.5 Morphological evaluation and biological performance of 3D biphasic systems

Biphasic hydrogels developed by enzymatic assembling of the mineralized and anisotropic features were assessed by μ-CT analysis to evaluate the integration between phases. As shown in the 3D reconstruction image (Figure 9A), a single continuous structure with well-integrated and smooth interface transition was successfully obtained. Interestingly, unlike other methods for obtaining biphasic scaffolds proposed for the regeneration of interfacial tissues, the remarkably simple and reproducible manufacturing method herein proposed, based on direct modelling without the need of inter-phase coating process, resulted in the effective integration between the interface [18].

To evaluate the potential of the biphasic system to recreate the dissimilar physical and biological functions of tissue interfaces, a single hydrogel construct with hASCs embedded into both sides of the integrated phases was prepared and cultured for 21 days. A transition in the morphological cellular arrangement could be appreciated by staining of the nuclei and the cytoskeleton and the subsequent analysis using confocal microscopy (Figure 9B). Whereas randomly distributed cells without preferential orientation were predominant in the mineralized phase, a clear cell alignment corresponding to the anisotropic section was displayed, as observed in monophasic hydrogels. These qualitative characteristics were confirmed by analyzing the directionality of the actin filaments (Figure 9D). Thus, a main sharp peak was obtained for the anisotropic phase distribution, but multimodal flat distribution was achieved in the case of cells present in the mineralized phase. Moreover, these differences observed in cell morphology are also reflected in their respective nuclei aspect ratio, which is higher for cells in the anisotropic section (Figure 9C).

On the other hand, specific markers previously assessed onto independent hydrogels units were also evaluated to further confirm their potential to induce distinct cell differentiation trends guided through the defined chemical and structural composition, as depicted in the Figure 9E-H. As expected, the biphasic constructs show a gradient of OPN and TNC deposition. Interestingly, whereas higher expression of the osteogenic marker OPN is observed in the section corresponding to the hydrogel reinforced with HA, the TNC deposition was more prominent in the anisotropic phase. These results demonstrate that the functionalities of the gelatin hydrogels tuned by adapting their composition and manufacturing conditions were maintained in the integrated biphasic construct.

Actually, the potential to encapsulate cells within the biphasic hydrogel network is one of the main advantages offered by the proposed strategy. In this sense, it is worth mentioning that the incorporation of cells in biphasic hydrogels with anisotropic regions previously prepared by directional freezing technique is limited to 2D seeding on the surface of the preformed structures, due to the not cell-friendly conditions required for their production [15]. Consequently, the size and distribution of the pores of the system inhibit the cell infiltration and ECM protein deposition, as recently observed for hASCs seeded onto biphasic silk fibroin scaffold with anisotropy gradient of porosity prepared by the freeze-casting method [44]. Moreover, despite many attempts have been made for the development of monophasic anisotropic hydrogels produced trough ice-templating technique [45,46], few designs have managed to effectively integrate dissimilar features gradients into a single unit [44,47]. Additionally, the potential for *in vivo* injectability and the possibility of the *in situ* exposure to a magnetic field may help to promote the clinical translation of enzymatically crosslinked hydrogels for interfacial tissue engineering purposes.

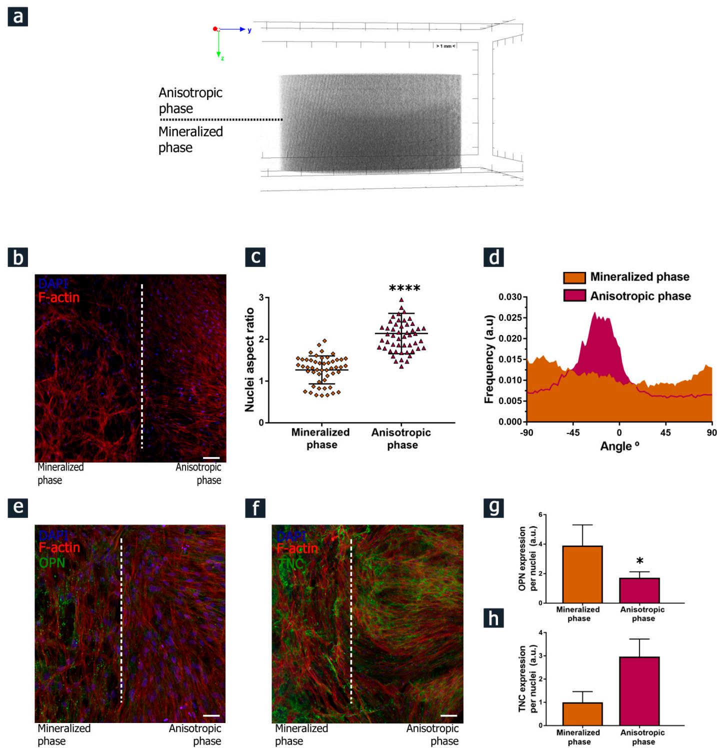


Figure 9. Morphological evaluation and biological performance after 21 days of culture of biphasic hydrogel compounded of mineralized and anisotropic phases. (A) 3D reconstruction from micro-computed tomography images of the biphasic hydrogel. (B) Representative confocal image of biphasic hydrogel´s interface with hASCs nuclei in blue and F-actin filaments in red after 21 days of culture. (C) Nuclei aspect ratio determination of nuclei from both, mineralized and anisotropic phases. (D) Directionality frequency plots of cytoskeleton alignment analysis. Osteogenic differentiation-related marker osteopontin (OPN) (E and G) and tendon tissue-related marker tenascin (TNC) expression evaluation in each of the different phases (F and H) (scale bars = 100 μm). Statistical differences: * $p<0.05$ and **** $p<0.0001$.

In general, engineered constructs aimed at tissue engineering must incorporate in their design requirements the use of non-complex and reproducible preparation methods, be easy to sterilize and cost effective, among others to move forward to clinical translation [48]. Herein, a simple and reproducible processing method to fabricate a single scaffold with well-integrated phases is reported. Besides, it is especially important to bear in mind that gelatin, the main material used for the preparation of the structures, is already approved for human use. [18]. Furthermore, unlike many anisotropic hydrogels prepared by the alignment of magnetic nanomaterials under exposure of magnetic fields of the same order of those used in this work (hundreds of mT), the alignment of CNC in the hydrogel network without requiring magnetic iron oxide nanoparticles simplifies the synthesis and could improve the safety profile of the biomaterial [33,49,50].

Overall, given the structural and compositional similarities of the biphasic hydrogels developed in this work with the intrinsic properties of the native enthesis, the tendon/ligament-to-bone interface, their potential application in regenerative approaches of this complex tissue can be envisioned. Indeed, this specialized tissue interface where tendons/ligaments are integrated in bones is characterized by progressive transition in collagen fibers organization and mineral and proteoglycan composition [51].

To further increase the potential of the proposed system to boost the healing process of such complex tissue, the nanocomposite hydrogel could be functionalized with growth factors in order to stimulate the stem cells differentiation towards the specific phenotype [52]. Despite the optimal combination of these biochemical factors is not fully understood, the gradual combination of bone morphogenetic protein 2 with fibroblast growth factor 2 [53] or transforming growth factor β2 with growth/differentiation factor 5 [54] have led to promising results. In this regard, although the evaluation of the incorporation of specific growth factors was beyond the scope of this work, the transglutaminase enzyme proposed in this research work presents the potential to support the covalent immobilization and the required sustained release of peptide growth factors [55,56].

Additionally, the use of multi-chamber bioreactor device could be an interesting approach to provide each region with customized and graded culture medium. Thus, the specific cellular environment may guide the differentiation of hASCs for *in vitro* applications.

4. CONCLUSIONS

In this study, we successfully developed an integrative 3D system, in which chemically and morphologically different sections have been assembled in order to generate gradients of mineralization and cellular alignment. Enzymatically crosslinked gelatin hydrogel served as the base material, incorporating fillers such as HA and CNC to provide advanced functionalities. While the inclusion of CNC and exposure of uniform magnetic field (400 mT) resulted in anisotropic structures that allow cell alignment and ECM protein deposition related to tendon tissue, the presence of HA particles generated stiffer hydrogels with feasibility to induce the differentiation of hASCs to osteogenic lineages. The design and versatility of these systems could be derived in both injectable formulations and implantable 3D structures to different tissues. Overall, this methodology could be potentially useful for the development of unique 3D systems with mineralization and/or cellular organization gradients that are specially interesting for engineering interfaces such as connections between fibrous, cartilaginous and calcified connective tissues of the musculoskeletal system.

5. ACKNOWLEDGEMENTS

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Discussion

According to the World Health Organization, musculoskeletal conditions comprise several disparate diagnoses that affect the locomotor system considered as greatest cause of disability worldwide (1). The locomotor system is made up of bones, muscles, joints and associated tissues such as tendons and ligaments, and provides form, support, stability and movement to the body. Some of the most common and disabling conditions that have the greatest impact on society include bone fractures and joint-related pathologies. Tendon and ligament injuries account for approximately 30% of all musculoskeletal consultations, presenting approximately 4 million new incidents annually worldwide (2). On the other hand, it is estimated that only osteoporosis causes more than 8.9 million fractures annually worldwide (3). Moreover, the impact of musculoskeletal disorders on individuals and society is expected to increase dramatically because of the predicted ageing of the world´s population. As a consequence, by 2050, the worldwide incidence of hip fracture in men is projected to increase by 310% and by 240% in women, compared to rates in 1990 (4).

Bone tissue uniquely heals upon a fracture without formation of scar tissue. In this way, when a bone lesion appears, the osteogenesis mechanisms are immediately initiated in order to restore the bone tissue at the site of the lesion. The repair process must restore the original geometry, the biology matrix and the biomechanical properties of damaged bone tissue (5). To achieve this regeneration, the involvement of the angiogenesis and osteogenesis processes are necessary, both stimulated by a combination of cytokines, growth factors, hormones, cells and extracellular matrix (ECM) components (Figure 1) (6). However, there are situations in which the capacity for regeneration of the tissue is insufficient and unsatisfactory as a method of healing. It is estimated that close to 10% of the bone fractures are unable to heal on its own (7). Pathological conditions such as osteoporosis or osteonecrosis are often involved in those conditions. Currently, the gold-standard treatment is performing an autologous bone graft surgical replacement. Nevertheless, this procedure is frequently not possible or not successful because the limited amount of available tissue and the high associated morbidity (8).

Conversely, the scenario is more challenging in the case of pathologies located in the tendon/ligament-to-bone insertions or enthesis. These highly heterogeneous and specialized interphase tissue regions integrate tendon and ligaments with bones and stabilize joints facilitating motion. Structural gradients of ECM composition, collagen molecule alignment and mineralization characterize the enthesis (9). Collagen molecule alignment increases gradually towards the tendon/ligament, whereas mineral content increases towards the bone tissue (Figure 2). These graded structural features are essential for their function; the smooth transfer of mechanical stresses between tendons/ligaments and bone (10). However, these specialized complex regions present poor ability to promote the restorative processes upon injury or in diseases characterized by degeneration progress.

In these cases, a fibrotic scar tissue with considerably different structure and composition from their native graded state is developed, often failing to fulfill their crucial mechanical functions and leading to a high number of rupture recurrence rates (11). The long term tissue functionality and clinical outcome are compromised in these situations. The gold standard approach to clinical management of such injuries is the surgical application of grafts. Despite recent advances in surgical techniques, interface repair still fails up to 95% of the time due to the formation of fibrotic tissue (12).

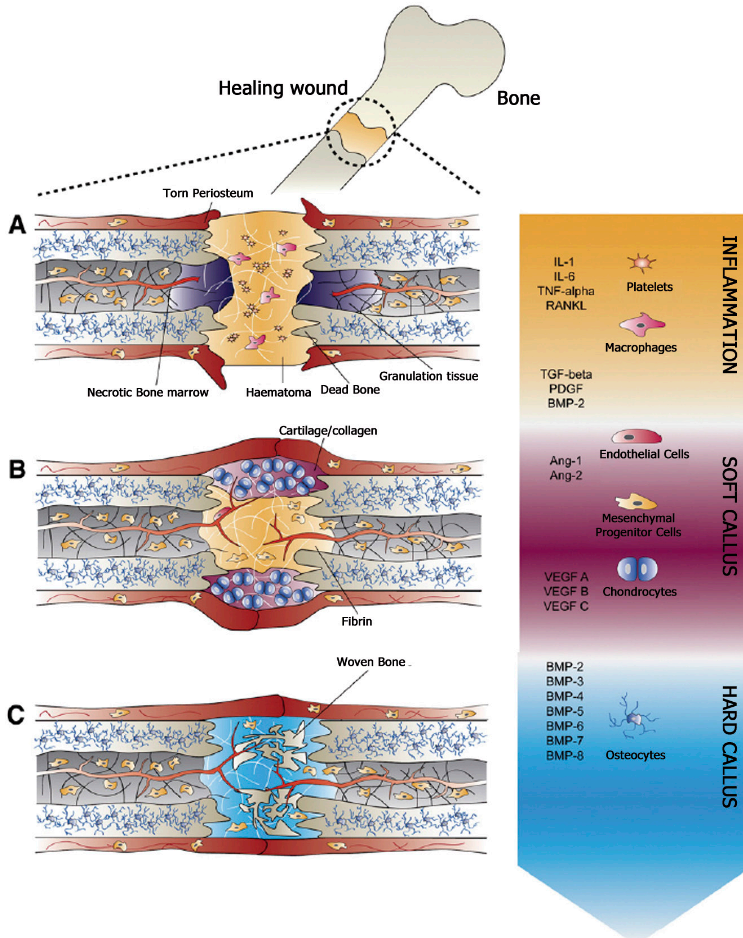

Figure 1. Bone regeneration during fracture repair. The bone healing process is categorized into three stages: (A) inflammatory phase, (B) Angiogenesis promoted soft callus formation, (C) hard callus formation by osteogenic differentiation of recruited mesenchymal progenitors. Each step is regulated by a myriad of soluble and matrix immobilized growth factors, matrix cues and different cell types. Figure reproduced from (6) with permission.

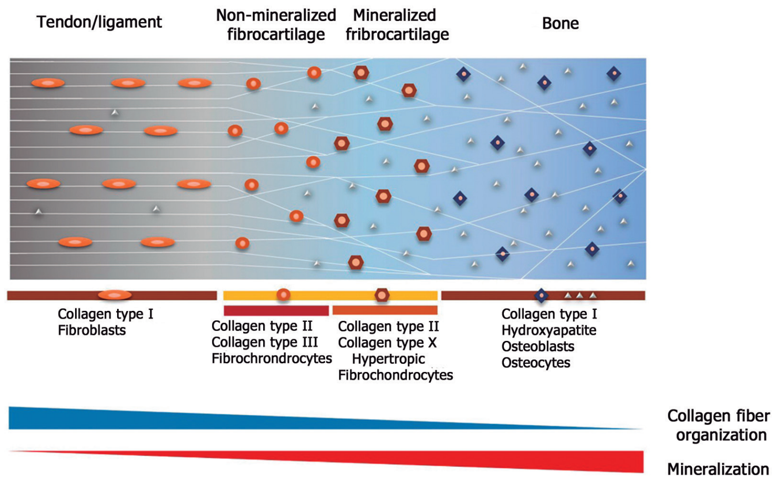

Figure 2. Composition and structure of fibrocartilaginous enthesis. The main extracellular matrix components and cellular types of each zone are indicated. Collagen fibers organization and mineral contents are reversely graded across the structure. Figure reproduced from (9) with permission.

The raised problems of tissue availability and/or rejection by the patient are common to any type of replacement surgeries. Thus, an immense effort has been made in order to develop novel therapies that could shed light on these challenges, specially under the umbrella of a new field known as Tissue Engineering (13). The latter is an emerging multidisciplinary research field focused on developing functional biological substitutes that repair, replace, maintain or improve the particular function of an organ or tissue to provide potential solution to injured tissues. This therapeutic arsenal is based mainly on the use of signaling molecules, cells and biomaterials. The role of tissue engineering in this context represents a new step forward in the evolution of substitute treatments. It is not so much about repairing, but about regenerating, of stimulating mechanisms already present naturally in the organism to give a clinically applicable response thanks to the collection of knowledge available in different disciplines of science. Therefore, knowledge on both physiology and pathophysiology of the injured tissues is extremely essential for the optimal development of these strategies.

Regarding the requirements of biological substitutes designed in the scope of tissue engineering, some general features are common for all approaches (14, 15). Thereby, the designed substitutes should be biocompatible, performing the repair function in the host tissue without eliciting any immune response and biodegradable at a rate in concert with neotissue growth. Moreover, highly porous structures with interconnected porosity are essential for the cell ingrowth, mass transport and neovascularization process. Beyond these properties, particular features are necessary to address bone defects (16) or injuries of the tendon-to-bone interface (17). In fact, while osteoconductive property is of major significance for biomaterials aimed at

bone tissue engineering, the emulation of complex and multiphasic structure of the enthesis is the main concern for interfacial tissue engineering approaches. Besides, the feasibility for serving as carrier for therapeutic agents, such as growth factors or even cells, is desirable for biomaterials designing (18).

Among all the materials that have been explored in recent years to engineer temporary structures that support regenerative processes, natural origin biodegradable polymers have increased popularity. The main advantage of these materials lies on their ability to support tissue growth and remodeling over their functional lifespan before being resorbed by the body. Gelatin is one of those promising materials with bioactive potential capable of interacting with the host tissue that has attracted a great deal of interest for both scaffolds and drug delivery carriers designing (19). However, effective crosslinking strategies to achieve stable networks at physiological temperature while preserving the intrinsic properties of this material are still required.

Unfortunately, considering all aspects that the designed substitute must fulfill for mentioned tissue engineering approaches, it is practically impossible for a single material to meet all the requirements. Thereby, combination of different materials in order to achieve composites with synergistic effect in their resultant properties has been explored as alternative (20). Nevertheless, designing ideal composites containing a minimal number of components in order to reduce the complexity and simplify the regulatory approval process remains a challenge. For instance, natural origin polymers have been combined with other materials such as ceramics to overcome the weak mechanical properties and the limited capabilities of pure polymers to integration with bone.

Bearing these considerations in mind, the current doctoral thesis was focused on the exploration of the gelatin potential as biomaterial to develop both biocompatible 3D systems for bone tissue regeneration and biphasic hydrogels recapitulating the complex tissue organization of the native tendon-to-bone interface.

In a first step, we aimed at designing gelatin strands crosslinking protocol to obtain 3D scaffolds for serving as both cell substrate and drug delivery system. For that purpose, microbial transglutaminase (mTG) enzyme derived from *Streptoverticillium mobaraense* was tested to induce the stable covalent union between amino acids of gelatin. This enzyme catalyzes an acyl transfer reaction between γ-carboxamide groups of glutamyl residue and ε-amino groups of lysine residue, which leads to the formation of crosslinked ε-(γ-glutamyl)-lysine isopeptide bonds. Unlike mammalian origin transglutaminases, one of the most outstanding advantages of this enzyme is the ability to catalyze inter and intra molecular crosslinking reactions under physiological conditions (37 °C, pH 7.4 and aqueous medium) in the absence of calcium ions (21).

Taking advantage of this property, through the crosslinking with this enzyme there is the possibility of preparing both cell-free gelatin 3D systems and gelatin hydrogels with encapsulated cells (22). In our case, in the first attempt cell-free gelatin 3D scaffolds were developed by freeze-drying technique using three different concentrations of gelatin (10, 15, 20 % (w/v) and three crosslinking degrees (10, 20, 30 U/g gelatin). The first characterization tests showed that the gelatin/mTG ratio investigated influences both the ability to swell after water absorption and the mechanical compression properties. Thus, the higher the concentration of gelatin and the enzymatic activity, the lower the swelling ratio of the system (Figure 3a). That effect was probably originated by the dense polymer network and consequent impediment of the water permeability (21). Inversely, the stiffness of the structures was significantly higher when the concentration of the gelatin and the level of crosslinking was increased (Figure 3b). These results show the feasibility for tuning the essential characteristics of the 3D scaffolds by adapting the gelatin concentration and the catalytic activity of the mTG enzyme.

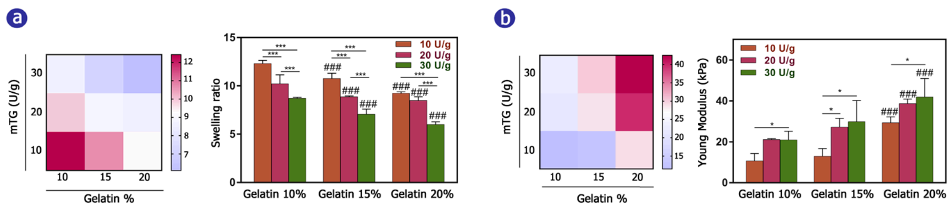

Figure 3. Swelling behaviour (a) and mechanical properties characterization under compression force (b) of the enzymatically crosslinked gelatin 3D scaffolds. Statistical significance: * $p<0.05$, ** $p<0.001$ and *** $p<0.001$ compared to the groups with the same concentration of gelatin and # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ compared to the scaffold with the same enzymatic activity and gelatin 10 % (w/v).

Subsequently, considering the high capability to absorb water of the scaffold GEL_10/20 and the stiffer properties of the GEL_20/20 system, further *in vitro* characterization was completed with those systems. To evaluate the morphology and microporous structure of the systems, scanning electron microscopy (SEM) images were acquired from the surface and the transverse section of the samples. In both cases, sponge-like isotropic structures with pores distributed throughout the entire surface were successfully fabricated demonstrating the usefulness of the freeze-drying technique to produce porous gelatin 3D systems (23).

Our next goal was to prove the cellular biocompatibility of these systems and to perform the biological performance evaluation for serving these systems as cell substrates. For the biocompatibility assessment, we followed the ISO 100993 guidelines for biological evaluation of medical devices and all the developed scaffolds were considered biocompatible, since all the samples presented viabilities above 70% in the direct and indirect cytotoxicity assays.

However, the lower viability values recorded in the direct test for all the samples suggested the adhesion of cells to the scaffolds through the interaction of integrins from the cell cytoplasmic membranes and the RGD sequences of the gelatin. To further investigate the capability of enzymatically crosslinked 3D scaffold to promote cell adhesion and proliferation, three cell lines previously used in the bone tissue engineering researches were selected as model cells (24, 25). Metabolic activity determination and staining of the nuclei at different times revealed that cells seeded on the scaffolds could proliferate properly, suggesting that the constructs developed in this work were suitable as cell-support structures. In addition, to assess the significance that these scaffolds may have in the bone regeneration environment, we examined the ability of these systems to either stimulate osteogenic differentiation or maintain the undifferentiated status of the murine bone-marrow derived mesenchymal stem cells (mBM-MSC). Interestingly, when these cells were seeded and cultured for 10 days, the stemness marker genes (*Oct4, Nanog*) expression were reduced while osteoblastic gene expression (*Col1a1, Runx2, Osx*) levels were significantly increased, especially when the culture medium was supplemented with the osteoinductive growth factor BMP-2 (Figure 4a).

Thereafter, the intracellular signaling triggered by the cell culture on the developed 3D scaffolds was analyzed in order to determine the cellular mechanisms involved in the BM-MSC differentiation process. After 10 days of culture, the decrease of phosphorylated ERK1/2 levels and the increase of phosphorylated S6-kinase and p38 confirmed that proliferation rate of the cells was reduced and their specification into a osteogenic program was promoted (Figure 4b) (26, 27). In fact, the phosphorylation of p38 within the activation of the non-canonical BMP signaling and the phosphorylation of the S6 kinase as an effector in the mTOR signaling pathway have been previously proved as molecular processes activated during the osteogenic differentiation of MSCs(28, 29).

Finally, to evaluate the potential of the developed gelatin scaffolds to absorb therapeutically active signaling molecules and support thereafter the sustained delivery of such factors, vascular endothelial growth factor (VEGF) and bone morphogenetic protein – 2 (BMP-2) were loaded on the systems, and *in vitro* release assays were carried out. These two growth factors were chosen as model molecules because of their role in bone remodeling (30, 31). VEGF is an angiogenic growth factor that is involved in the biological processes of bone healing and its main function is based on increasing the permeability of sinusoidal endothelial cells to induce neutrophil migration during the inflammation phase of the remodeling (32). On the other hand, as previously mentioned, the BMP-2 is one of the most potent osteoinductive factor, currently approved for clinical use on non-unions long fractures and lumbar spinal fusion treatments (33).

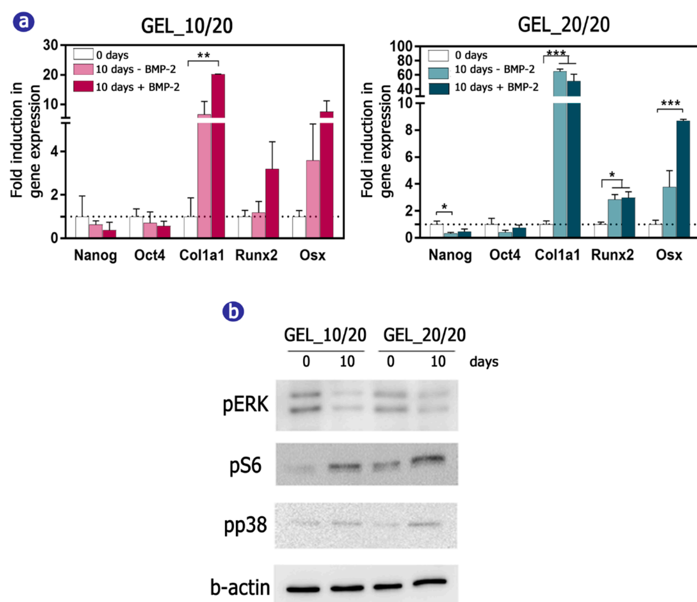

Figure 4. Scaffolds increase osteogenic marker expression. (A) Primary BM-MSCs were cultured on GEL_10/20 or GEL_20/20 scaffolds with or without 2 nM of BMP-2 for 10 days. The mRNA expression of *Nanog, Oct4, Col1a1, Runx2* and *Osterix* was analysed and normalised to *Tbp* levels. (B) Western blot analysis of the phosphorylation levels of ERK, S6 and p38. Cells were cultured in the scaffolds for 10 days. Data was quantified relative to the levels of b-actin. Differences were considered significant at *p* values: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ when compared to control cells at time 0.

High encapsulation efficiencies were achieved for both factors and approximately 90% of the loaded dose was absorbed in the scaffold. The ionic complexation between the gelatin strands with isoelectric point (IEP) between 4.7-5.2 and the growth factors (IEP VEGF: 8.5 and IEP BMP-2: 7.6) allowed both the absorption and the release of the factors throughout the assay. In all cases, the release profiles were successfully fitted to the first-order kinetic model, suggesting that the release of bioactive molecule is proportional to the quantity of the drug remaining in the dosage form (34). Although these 3D systems showed a burst release effect of around 50% in the first 24 hours, the GEL_20/20 scaffold presented greater capacity for the retention of the growth factors, given to the greater amount of gelatin for electrostatic interactions between negatively charged amino acids and growth factors (Figure 5) (35). Moreover, the experimental cumulative percentage released at the end of the assay corresponds to % $VEGF_{released\ \infty}$ and % $BMP\text{-}2_{released\ \infty}$ parameters values estimated with the non-linear regression fitting. These results suggest that complete release of the factors by diffusion was achieved during the time of the testing and the rest of the unreleased protein could be delivered by means of scaffold degradation under real physiological conditions (36).

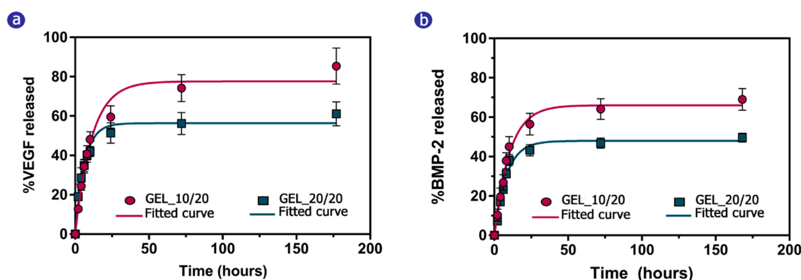

Figure 5. *In vitro* release of vascular endothelial growth factor (VEGF) (a) and bone morphogenetic protein -2 (BMP-2) (b) from enzymatically crosslinked gelatin 3D scaffolds. The experimental data of the cumulative release and the fitted curve to first-order release kinetics are displayed for each growth factor.

Taking into account all these findings, we considered that the scaffold GEL_20 / 20 would be the best candidate for further explore the potential to design gelatin composites biomaterials for bone tissue engineering. Despite suitable characteristics for cell-adhesion support and drug delivery were noticed, it is necessary to improve the intrinsic osteoconductive properties of the 3D scaffolds in order to reduce the supraphysiological doses of BMP-2 employed until now and relieve the side effects associated with high dosing levels.

In the second step of the present thesis, we focused on the development and characterization of gelatin-based composite biomaterials with tailored features for bone tissue engineering, with special focus on particularly challenging bone repair conditions, such as osteoporosis disease. For that purpose, organic/inorganic composite 3D scaffolds which better mimic the composition of the native bone were developed. The formulation optimized in the first work (GEL_20/20) was employed as the organic component and calcium bioceramics hydroxyapatite (HA) and calcium sulfate were chosen for the inorganic phase. Although these materials have previously been used as bone void fillers, some limitations such as the poor bioresorbability of HA, the challenging stable surgical fixation on these stiff/brittle substitutes and the lack of vascularization in their central part has been associated with them (37).

First, we reinforced bioceramic materials by gradually incorporating them within the gelatin networks. As a consequence, three levels of reinforcement were designed and the effect of the inorganic phase presence on swelling, degradability and mechanical properties of the scaffolds was explored. Despite all prototypes showed great ability to water uptake, the swelling ratio decreased proportionally with the level of reinforcement (Figure 6a). On the other hand, the presence of inorganic salts with different hidrosolubility affected the *in vitro* hydrolytic degradation. Although all the developed systems showed biphasic hydrolytic degradation profile probably due to physical bulk erosion through the diffusion and dissolution of oligomers, the scaffolds reinforced with apatite ceramic revealed better water stability than

scaffolds reinforced with calcium sulfate (38). Moreover, all the scaffolds were degraded by collagenase enzyme, suggesting the biodegradability of the scaffolds after the *in vivo* implantation in presence of ECM remodeling proteases (39). Consequently, the expected release of calcium ions from the composite scaffolds to the surrounding environment, may promote potentially a chemotactic paracrine effect to induce the recruitment of endogenous osteoprogenitor cells and promote the osteogenic differentiation (40).

Regarding the mechanical properties, uniaxial static compression tests were performed to determine Young and Aggregate modulus for each system (Figure 6b). The Aggregate modulus determined from the confined tests were higher than the Young modulus in all the prototypes. These results indicate that the scaffolds generate a lateral expansion during the unconfined axial compression (41). The incorporation of bioceramics resulted in reinforcing effect compared to bare gelatin scaffold, being in the case of calcium sulfate this effect proportional to the included concentration, but not in the case of HA incorporation.

In this regard, there is no a consensus about the optimal stiffness of implantable 3D polymeric scaffolds for bone tissue engineering. However, there are several studies demonstrating that hydrogels with Young modulus around 60 kPa induce the osteogenic process of MSC both *in vitro* and *in vivo*, whereas higher values of Young modulus (126-181 kPa) have been associated with improved surgical handling and stable graft fixation rates (42, 43). All these findings suggest that the enzymatically crosslinked gelatin network could be easily tuned to achieve tailored properties by incorporation of calcium-based bioceramics. Therefore, we considered that the developed gelatin-based composite scaffolds gathered adequate essential characteristics to continue deepening the characterization and evaluation of the therapeutic potential. Nevertheless, we limited the further *in vitro* characterization, biological commitment evaluation and *in vivo* bone regeneration performance assessment to scaffolds composited of 7% of calcium mineral elements and bare gelatin scaffold as control group.

Microstructure and chemical characterization of the prototypes were completed by means of SEM images, energy dispersive X-ray spectroscopy (EDAX), fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis. The EDAX mapping performed on the freeze-dried samples revealed that while calcium element was observed in both reinforced scaffolds, sulfur and phosphorous elements were reported in calcium sulfate and HA reinforced systems, respectively. Moreover, FTIR and XRD spectrum (Figure 6c,d) revealed the particular peaks corresponding to HA and calcium sulfate in developed composite scaffolds, confirming the presence of reinforcing minerals after the freeze-drying procedure. Interestingly, the SEM images showed that porous structures were developed and the inclusion of both reinforcing materials resulted in increase of pore size, especially with HA incorporation (Figure 6e). The pore area determined in the gelatin/HA composite scaffold

(around 100 µm) is proven to promote the migration and proliferation of osteoblasts, thereby providing interesting features for bone repair process (44).

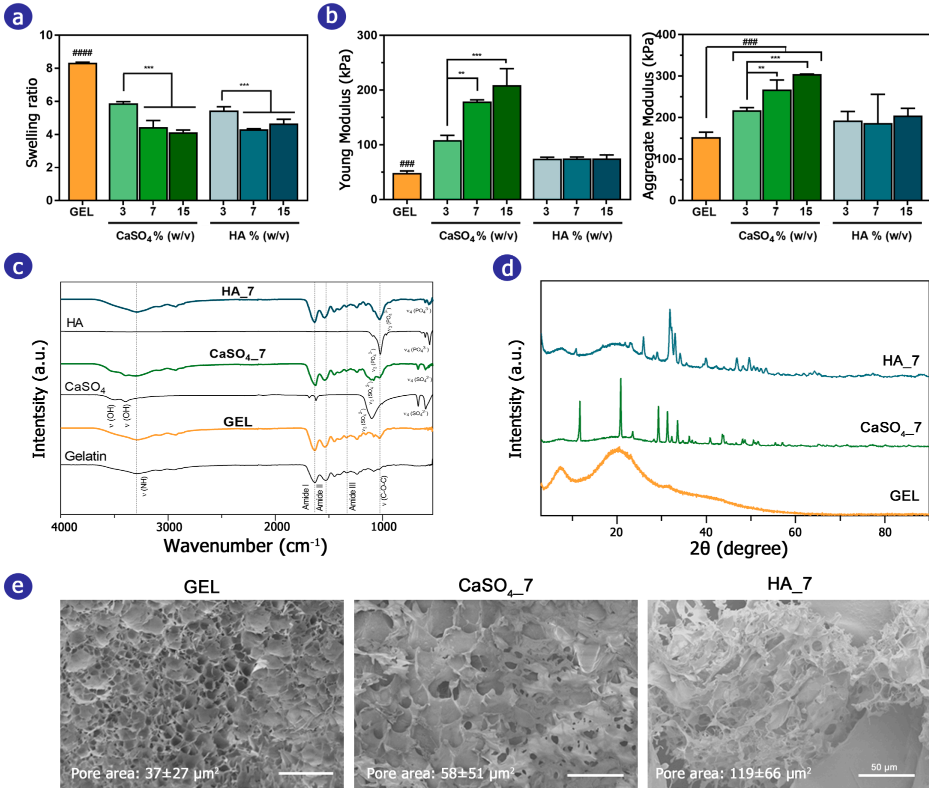

Figure 6. Physic-chemical characterization of developed gelatin/bioceramics composite scaffolds. (a) Swelling ratio and (b) compressive mechanical properties determination of gradually reinforced systems. FTIR spectrum (c) and XRD patterns (d) of developed composite scaffolds. (e) Representative SEM images of lyophilized scaffolds. HA: hydroxyapatite. Statistical significance: ** $p<0.01$ and *** $p<0.001$; ### $p<0.001$ and #### $p<0.0001$ compared GEL to all the reinforced scaffolds.

Subsequently, before the assessment of *in vivo* bone repair potential of the developed scaffolds, *in vitro* biological performance was evaluated with human bone marrow-derived stem cells (hBM-MSCs). These primary cells have been broadly used to determine the *in vitro* osteoinductive properties of 3D scaffolds because of their easy accessibility, high osteogenic differentiation ability and the elevated replicative potential, among others (45). First, preliminary biocompatibility assays were carried out following ISO 10993 guidelines as previously described. Although some toxic effect have been earlier reported with calcium phosphate-based biomaterials due to high ionic reactivity causing changes in the ionic composition of the culture medium (46), all experimental viability values from the developed

gelatin composite systems exceeded the threshold limit established for non-cytotoxic biomaterials. To evaluate the ability of scaffolds to support hBM-MSCs adhesion, proliferation and osteogenic differentiation, cells were seeded on the surface of the preformed scaffolds previously rehydrated with the culture medium and culture basal medium was used to maintain the cell culture. To assess the cellular viability, live/dead staining was performed 2 and 10 days after the seeding. Immunofluorescence images and flow cytometry analysis showed that cells maintained high rates of viability on the structures without significant differences between experimental groups. When analysing the cytoskeleton and nuclei of cells by fluorescence staining, we observed that cells were completely adhered and spread on the surface of the scaffolds (Figure 7a).

These results indicate that the incorporation of bioceramic fillers does not affect to the interactions between the RGD motifs of the gelatin and integrin receptors of the cell membranes. Metabolic activity analysis determined that cells proliferated specially within the first week post-seeding without notable differences between the developed composite scaffolds (Figure 7b), which suggested that suitable platforms to support cellular activities were developed. Given that these anchorage-dependent cells need to adhere to the surfaces to maintain both the viability and osteogenic potential, we hypothesized that the capacity for osteogenic differentiation of hBM-MSCs could be maintained or even promoted on the developed structures (40).

To prove such an hypothesis, our next goal was to assess the fate of hBM-MSCs seeded on the developed 3D scaffolds by means of stemness and osteogenic commitment markers expression evaluation. The activity of secreted alkaline phosphatase (ALP) and the expression levels of five genes were determined (47, 48). Although the expression levels of stemness genes (*Nanog, Oct4*) were downregulated over the time for all the scaffolds and the ALP activity of the cells seeded in the organic/inorganic composite scaffolds increased throughout the culture period (Figure 7c,d), confusing results were obtained with osteogenic markers expression analysis. The results from the RT-PCR assay revealed upregulation of *Col1a1* and *Fmod* osteogenic marker gene expression especially on gelatin/HA scaffold, whereas the expression of the main osteogenic transcription factor *Runx2* remained stable for all the experimental groups.

Despite this transcription factor is considered the key regulator in osteogenic differentiation of MSCs through several molecular routes such as canonical and non-canonical BMP, Wnt/β-catenin or ERK1/2 signaling pathways (28, 48), the exploration of other signaling routes and molecular mechanism involved in the regulation of the osteogenic differentiation are needed (49, 50). For instance, some recent studies have concluded that the direct interaction between *Osterix* transcription factor and NFATc1 cofactor can promote the activation of the *Col1a1* gene promoter without activating the *Runx2*-dependent transcription (51, 52).

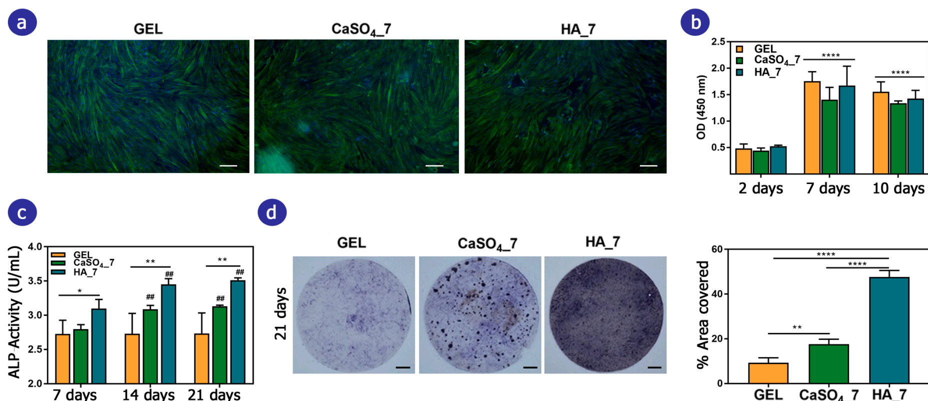

Figure 7. Evaluation of human bone-marrow derived mesenchymal stem cells (hBM-MSCs) adhesion, proliferation and osteogenic commitment after cultured on the developed scaffolds. (a) Fluorescence microscopy images of hBM-MSCs stained for nuclei (blue) and F-actin (green) after 7 days of culture (scale bars= 200 µm). (b) Metabolic activity of the cells cultured on the scaffolds for 10 days. (c) Secreted alkaline phosphatase (ALP) activity after 7, 14 and 21 days of culture. (d) ALP staining images and the relative quantification of the stained area after 21 days of cell culture (scale bars = 1.5 mm). Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; ## $p<0.01$ compared with the same scaffold at day 7.

Together, our data suggest that, the developed composite 3D scaffolds may affect the differentiation process of hBM-MSCs, probably by promoting the osteogenic lineage commitment but further research should focus on the mechanism involved in such effect, in order to gain knowledge over the interactions between these cells and the developed biomaterials. We next focused on the potential of proteomic analysis to explore the cellular and molecular events involved in the interactions between developed scaffolds and cells seeded on their surface.

Unlike other techniques suitable for investigating only the action of a limited number of proteins, the liquid chromatography coupled to tandem mass spectrometry is considered a powerful tool for the large-scale characterization of proteins offering the possibility for the global and integrated evaluation of the entire proteome (53). Nevertheless, to get valuable data and conclusions it is essential to optimize the conditions of sample processing and analysis methodology with particular emphasis on the cell/biomaterials tandem. Given the protein nature of our 3D scaffolds, setting the ideal conditions for the proteomic analysis may result especially challenging. To stablish the particular conditions for the acquisition and analysis of cells proteomic profile, *in vitro* cell culture conditions, protein elution protocols and two different mass spectrometry technologies were explored.

We observed that performing mild rinses of the scaffolds with PBS to discard proteins of the culture medium serum is a good starting point for the elution of cellular proteins. Then, cell lysis with cell lysis buffer (CLB) consisting on urea 7M, thiourea 2M and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) 4% was proven to be good strategy to extract proteins before tryptic digestion. Although bovine origin proteins from the developed scaffolds were also eluted with this protocol, the use of last generation timsTOF Pro mass spectrometer with unprecedented sensitivity and resolution allows to improve the number of identified proteins in each sample (several thousands of proteins were identified from each scaffold). Moreover, despite a commercial serum-free culture medium, specially formulated for the growth and expansion of hMSCs without any animal component, the amount of identified bovine proteins was not reduced.

However, since the ratio between bovine and human origin proteins was constant in all the experimental groups (approximately 80% of the total identified proteins were from human origin) we were able to proceed with the quantitative analysis of the samples. We used PEAKS® software for the identification of proteins and PEAKSQ module for the quantitative analysis. Only proteins identified with at least two unique peptides were considered during the process and intensity of each identified proteins was determined for each sample. To compare the proteomic profiles of cells seeded on different scaffolds, the intensities assigned to each protein and sample were analyzed by Perseus software. Thereby, proteins and samples were grouped based on their abundance similarity and we were able to conclude that scaffold composition in which the cells were seeded affect the proteomic profile of hBM-MSCs. Interestingly, Gene Ontology analysis or Ingenuity Pathway Analysis could allow us to gain knowledge over the biological functions in which the differential proteins of each experimental groups are involved. Overall, we successfully established the conditions for the proteomic analysis by mass spectrometry and we envision that performing the assay by increasing the number of samples and using cells from different patients can provide crucial information about the hBM-MSCs seeded on the developed gelatin composite scaffolds.

Having explored the biocompatibility of gelatin composite structures and studied the activity of hBM-MSCs on the scaffolds, our next step was to functionalize such osteoconductive cell-free platforms with osteoinductive therapeutic factor for drug delivery functions. In particular, we loaded the 3D scaffolds with BMP-2 by soak-loading procedure and preliminarily, we evaluated the *in vitro* release profile. The incorporation of calcium sulfate and HA in the formulation did not affect the innate ability of the gelatin to adsorb the growth factor and promote the sustained release. The first burst release of the 35-50% of loaded protein from the composite scaffolds was noticed within the first 2 days.

According to these results, the presence of bioceramics in the scaffolds generates a slight extension of the burst effect over time, probably due to the non-specific electrostatic

interactions occurred between the anionic groups of the sulfates and phosphate salts and the cationic groups of the BMP-2 (54). Subsequently, the release of the growth factor was sustained from both reinforced gelatin scaffolds, and around the 60% of the loaded dose was delivered in 2 weeks. We hypothesized that reinforced gelatin 3D scaffolds could serve as a carrier of this osteoinductive factor, improving the spatiotemporal control of the therapeutic agent in the injury to reduce both the administered dose and the adverse effects associated with the supraphysiological dosing.

To prove such hypothesis, our next goal was to study the bone regeneration potential of gelatin composite scaffolds loaded with low dose of BMP-2 in osteoporotic mice critical-sized calvarial defects. The animal model used to evaluate the ability of developed substitutes to promote bone repair directly affects the therapeutic behavior of the bone tissue engineering prototypes. For instance, disparate results have been achieved regarding the bone regeneration with same formulation loaded with BMP-2 in healthy and osteoporotic rats. Therefore, our strategy was to assess the bone repair potential of developed gelatin-based scaffolds in a particularly challenging animal model. In fact, the delay on bone repair associated with osteoporosis is further complicated in critical-sized bone defects due to diminish self-healing ability of the diseased bones. Moreover, the protocol for osteoporosis model induction that we applied has been previously validated, demonstrating altered structural features of calvaria bone (55). Developed scaffolds loaded with or without 600 ng of BMP-2 were implanted in 4 mm sized osteoporotic mice calvaria defects.

To analyze the therapeutic effect of the 3D scaffolds, the defect area was histologically and histomorphometrically evaluated 8 weeks after the implantation. Although new bone tissue and bone microarchitecture were noticed in all the experimental groups (Figure 8a, c), in the case of blank scaffolds the repair response was restricted to the margins of the defect. In contrast, 3D scaffolds loaded with BMP-2 promoted significantly the bone regeneration process and around half of the defect surface was occupied by newly formed bone. Promisingly, more compact structure, mineralized ECM regions and osteocytes-like cells were observed with the implantation of gelatin/HA scaffolds functionalized with BMP-2. Accordingly, higher relative expression level of the late osteogenesis and mineralization marker osteocalcin (OCN) was revealed with the implantation of composite scaffolds loaded with BMP-2 (Figure 8b,d), being significantly higher in the case of HA_BMP-2 group compared to $CaSO_4$_BMP-2 group.

Overall, we proved the potential of tailored gelatin 3D scaffolds by incorporation of bioceramics to serve as osteoinductive drugs carrier systems, promoting bone tissue regeneration in specially challenging diseased environment, such as critical-sized osteoporotic bone defects.

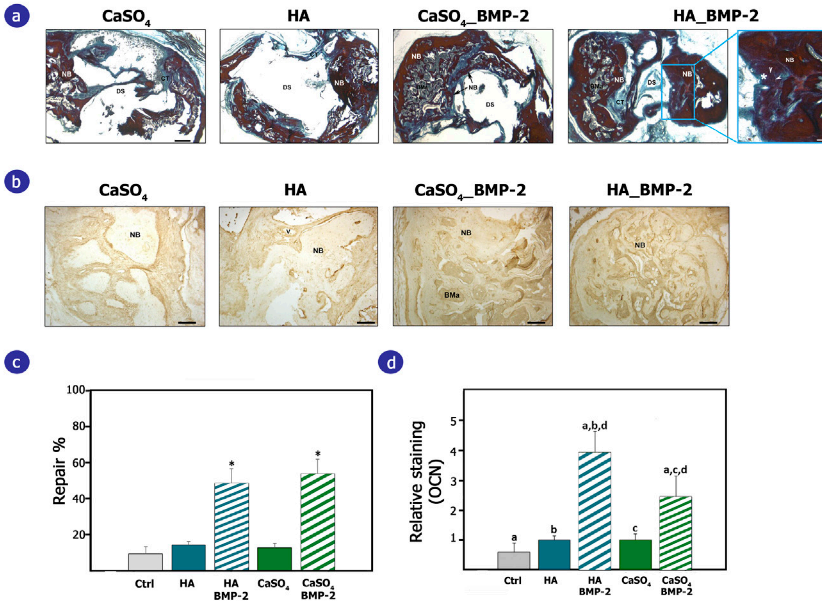

Figure 8. Bone regeneration assessment on osteoporotic mice calvaria defects 8 weeks after implantation of developed 3D scaffolds. (a) Representative VOF staining images in horizontal section showing the defect site in the different experimental groups. The region marked with the blue box stands out in the HA_BMP-2 group, which is shown at high magnification, shows an early mineralization zone defined as extracellular matrix of conjunctive-bone transition stained red (*) in which groups of osteocytes-like cells (arrowheads) are detected. Scale bars: Panoramic images = 500 µm. Detail image = 50 µm. (b) Representative images in horizontal section showing OCN immunoreactive staining in the defect site. Scale bar = 100 µm. (c) Quantitative analysis of histomorphometry showing the percentage of repair. (d) Relative OCN staining in arbitrary units in the different experimental groups. The same letters on different histograms indicates significant differences between these groups. BMa: Bone marrow, CT: Connective tissue, DS: Defect site, NB: Newly formed bone, v: Blood vessel. Statistical significance: $p<0.001$.

Taking into account the promising results obtained with the biomimetic gelatin/HA composite scaffold, in the next step of the thesis, we aimed at integrating the mineralized osteoconductive gelatin composite and bioinspired anisotropic hydrogel in a single construct, where biochemical and structural cues are spatially varied. This strategy is categorized within interfacial tissue engineering approaches, where the main objective is the development of structures that recapitulate the dissimilar composition, architecture and cellular organization found in the musculoskeletal interfaces, such as in the tendon-to-bone enthesis. The addition of functionalities enabling the modulation of specific components of a construct *ex vivo* strongly improves the design of smart biomaterials for tissue interfaces (56). Based on that assumption, we also employed gelatin as the main biomaterial in this attempt and specific nanometric and micrometric fillers were successfully combined with it to produce

tailored composites that simulate each of the phases. Thus, adapting the composition and manufacturing conditions of gelatin-based hydrogels was the strategy followed for that purpose. In a similar way that in the works carried out previously, the gelatin crosslinking was done with the mTG enzyme. In this regard, we hypothesized that the enzymatic crosslinking of the gelatin could serve not only to create stable structures under physiological conditions, but also as a method of assembling independent phases in a single construct. Moreover, the possibility to encapsulate cells inside of 3D enzymatically crosslinked gelatin hydrogels overcomes the important limitations related to other technologies for anisotropic hydrogels manufacturing. For instance, due to the not cell-friendly conditions required for the production of anisotropic systems by directional freezing, the incorporation of cells to these systems is limited to 2D seeding on the surface of the preformed structures (57). However, it has been shown that 3D cell culture may provide more realistic biochemical and biomechanical microenvironments and more accurate assessment of *in vitro* biological performance (58).

First, mineralized or anisotropic monophasic hydrogels were developed and *in vitro* characterization was performed, evaluating the biological performance with encapsulated human adipose tissue-derived stem cells (hASCs). For the mineralized phase, HA particles were added and SEM images showed that the microparticles were well-dispersed into the enzymatically crosslinked gelatin hydrogels (Figure 9a). The compressive mechanical testing revealed stiffening effect on the hydrogel with the incorporation of bioceramic particles and the Young modulus of the mineralized composite was twice higher than the pure gelatin hydrogel (Figure 9b). Interestingly, this value is in the same range of osteoid stiffness, the bone precursor crosslinked collagen secreted by osteoblasts (59). Next, to determine the potential of the mineralized system to guide osteogenic differentiation of encapsulated hASCs, the activity of the secreted ALP and the expression of bone-related marker osteopontin (OPN) by immunostaining were evaluated (60). The gradual increase in the ALP activity (Figure 9c) and enhanced expression of OPN (Figure 9 d,e) from cells encapsulated in the mineralized hydrogel confirmed the intrinsic capability to promote the differentiation of hASCs toward osteogenic lineage. In fact, the high level of this marker is associated with the process of ECM mineralization that comes up during the late osteogenic differentiation of osteoprogenitor cells (61).

After optimized the mineral phase formulation, we focused on the development of biocompatible hydrogels with anisotropic arrangement. In this vein, the mimicry of the distinct ordered ECM architecture and cellular organization own of the connective tissues such as tendons or ligaments is extremely important since these features play an essential role in the biomechanical and biological functions of these tissues (57).

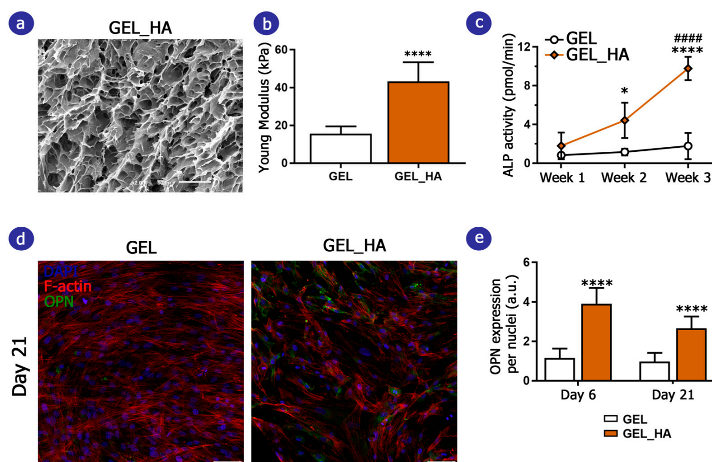

Figure 9. Physical and biological characterization of mineralized hydrogels. (a) Representative SEM image of gelatin_hydroxyapatite (HA) composite hydrogel. (b) Young Modulus of the hydrogels obtained under compression tests. Statistical differences: $p<0.0001$. (c) Determination of alkaline phosphatase (ALP) activity secreted by hASCs after 1, 2 and 3 weeks of culture. Statistically significant differences between groups at each culture time are shown as * $p<0.05$ and **** $p<0.0001$. (d-e) Confocal images and quantification of the Osteopontin (OPN) expression (green) at 6 and 21 days of culture with nuclei and cytoskeleton stained in blue and red, respectively. Statistical differences: **** $p<0.0001$ (scale bar = 75 μm).

With that purpose, cellulose nanocrystals (CNC) were incorporated in the gelatin hydrogel and the crosslinking process was carried out under the exposure to relatively low magnetic field strength. These rod-shaped nanoparticles extracted from the crystalline regions of the cellulose microfibrils have previously demonstrated their potential for tissue engineering applications. Excellent mechanical behavior, good biocompatibility and sensibility to relative orientation under magnetic field are some of the most important properties of these nanofillers (62). Our goal here was to manipulate CNC orientation within a hydrogel matrix using low strength magnetic fields despite their diamagnetic character (63). Two concentration of CNC (0.5 and 1% (w/v)) and two magnetic force levels (200 and 400 mT) during the crosslinking phase were applied for the preparation of anisotropic hydrogels (AnisoGEL). Gelatin hydrogels containing CNC and crosslinked without exposure of magnetic field were considered as a control (IsoGEL). For the exposure of the magnetic field, a custom-made magnetic system consisting of two neodymium permanent magnets was employed. To determine the strength of the applied magnetic field, a gaussmeter was used. We observed that by adjusting the distance between the magnets, different levels of uniform magnetic field was induced on the hydrogel deposited between them (64).

To evaluate if those conditions were enough to induce structural reorganization towards anisotropic arrangement, microstructure analysis by SEM, birefringence under polarized optical microscope and compressive properties evaluation were carried out. The internal organization of the nanoreinforced gelatin systems was assessed with the freeze-dried and cross-sectioned samples. Although porous structures were obtained in all cases, randomly oriented porous without any organization were noticed in IsoGEL systems, whereas more organized arrangement with larger pores were observed in AnisoGEL constructs. That was probably originated by the spatial reorganization of the gelatin strands. The directionality analysis of the SEM images confirmed the structural differences between groups, showing a frequency peak at a particular angle of orientation for AnisoGEL hydrogels containing both 0.5 and 1% CNC (Figure 10a-c).

Polarized optical microscope was used to determine the time of magnetic field exposure required to obtain aligned hydrogels. For that purpose, physical crosslinking of the gelatin was performed at different time intervals by cooling the samples on ice-bath. The continuous multi-chromatic transmission images showed by gelatin nanocomposite gels produced without external magnetic exposure demonstrated the presence of randomly organized multi-domain structure with heterogeneous birefringence. Conversely, a chromatic transition from multichromatic to monochromatic displayed in the case of AnisoGEL suggested the formation of mono-domain structure with homogeneous birefringence (65). Although all the AnisoGEL formulations displayed the chromatic transition before 15 minutes, the increase of the concentration and the magnetic field strength resulted in a faster alignment of the CNC. Hence, hydrogels containing 1% CNC and exposed to 400 mT magnetic field (AnisoGEL1_400) showed mono-chromatic image after 10 minutes. These results are in agreement with literature pointing out fast initial ordering of CNC suspensions within minutes in response to increasing magnetic field strengths (66). This time interval is completely permissible with the gelation time of the hydrogels, since the enzymatic crosslinking of the systems occurs at 20 minutes, similar to other injectable formulations designed for tissue engineering (67).

The presence of anisotropic microstructure in nanocomposite hydrogels exposed to a magnetic field during the gelation process was further confirmed with mechanical compressive properties analysis. The incorporation of nanofillers in the gelatin hydrogel promoted a reinforcing effect and the induction of CNC alignment increased more the compressive properties. As previously suggested by other authors, the existence of strictly arranged planes of aligned rigid CNC perpendicularly oriented to the course of compression might enhance the resistance to compression of the hydrogel (68).

Subsequently, to evaluate the effect of intrinsic nanotopographical features of the anisotropic hydrogels on cellular behavior and differentiation fate, morphological and biochemical

cues related to anisotropic tissues were evaluated. It has been previously described that the size, geometry or even the spatial organization of nanotopographical cues could have influence on various biological process of stem cells such as the adhesion, migration, proliferation, morphology and differentiation fate (64, 69, 70). These effects are essentially coordinated through mechanotransduction response and several molecular pathways are evolved for the transference of stimulus from ECM to the cellular nucleus (69). hASCs encapsulated in the nanocomposite hydrogels showed high viability, without significant differences between groups, suggesting that the developed gelatin composite hydrogels were suitable for cellular growth support.

To evaluate the effect of hydrogel alignment on cells cytoskeleton organization, F-actin staining was performed after 3, 7 and 21 days of culture and the cellular orientation was observed under confocal microscopy. The cells encapsulated in gelatin-based nanocomposite hydrogels displayed spindle-shape morphology and the increase of the cell density spread through the 3D environment suggested a suitable cell proliferation over time. Random organization without any preferential orientation was observed with cells embedded into IsoGEL systems, whereas the cellular alignment and preferential orientation in growth in AnisoGEL systems were noticed from day three and maintained after 1 week of culture. Interestingly, this effect was essentially an outcome of the intrinsic anisotropy of the hydrogels and that guidance to the oriented cellular growth was enough during the entire culture period, without the need of extra magnetic stimulation (64).

Additionally, hASCs encapsulated in AnisoGEL1_400 hydrogels for 21 days in comparison to those embedded in isotropic hydrogels presented elongated nuclei, higher expression of tenascin (TNC) per nuclei and and fibrillary and organized deposition of this glycoprotein, consistent with the cell alignment orientation (Figure 10d-f). These results indicate the potential of the developed anisotropic nanocomposite gelatin hydrogel to promote tenogenic commitment of hASCs (71). Regarding the influence of the magnetic field strength used to produce anisotropic hydrogels on cell organization, no significant differences were noticed between 200 or 400 mT exposed hydrogels. These results suggest that although the higher magnetic field used in this study leads to increased hydrogel physical anisotropy, it is not different enough to impact 3D cell organization.

Overall, we succeeded in simulating anisotropic environment found in tendon native tissue by designing magnetically responsive gelatin/CNC composites and tailoring the structural feature by low strength magnetic field exposure.

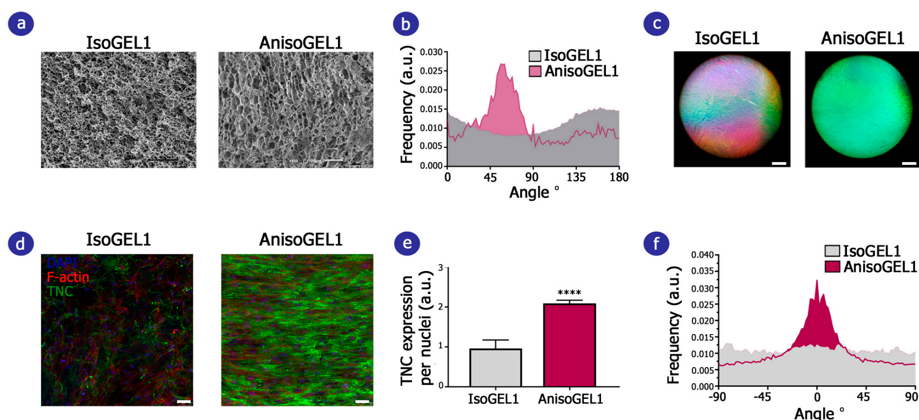

Figure 10. Effect of magnetic field exposure during the crosslinking to gelatin/cellulose nanocrystals composite hydrogels. (a) Morphological characterization by scanning electron micrographs (scale bar= 50 µm). (b) Structural directionality evaluation of isotropic and anisotropic hydrogels. (c) Polarized optical microscopy images of completely crosslinked hydrogels. (d-f) Tenascin (green) expression by human adipose tissue-derived stem cells encapsulated in nanocomposite 3D hydrogels after 21 days of culture. Normalized TNC expression and the directionality of deposition are displayed. Scale bar = 100 µm. Statistical significance: **** $p<0.0001$.

Having explored the biomimetic mineralized and anisotropic features in independent systems, our next goal was to integrate the tuned functionalities of the gelatin hydrogels in a single biphasic 3D system. Gelatin/HA composite was selected for the bone tissue mimicking mineralized phase and gelatin/CNC composite crosslinked under 400 mT magnetic field exposure was used to recapitulate the tendon tissue anisotropic structure. To evaluate the potential of enzymatic assembly of gelatin hydrogels to prepare fully merged structures, µ-CT analysis was performed. The 3D reconstruction image of the sample showed a single continuous structure with well-integrated and smooth interface transition (Figure 11a,b). This observation indicates that the presence of the remaining free reactive groups of gelatin in the pre-crosslinked hydrogel allows the contiguous crosslinking of a new hydrogel trough the catalytic activity of the enzyme. Importantly, remarkably simple and reproducible manufacturing method was suggested, avoiding any extra complex procedures previously proposed for the integration purposes (72).

We next prepared the biphasic structure with hASCs encapsulated in both sections to determine if the tailored functionalities of the gelatin hydrogels were maintained in the integrated construct. Cell-laden 3D system was cultured for 21 days and specific stains against cytoskeleton, nuclei, OPN and TNC were done. We observed that while randomly distributed cells without preferential orientation were predominant in the mineralized phase, a clear cell alignment corresponding to the anisotropic section was displayed (Figure 11d).

Additionally, the immunofluorescence images showed gradient of OPN and TNC deposition. Interestingly, whereas higher expression of the osteogenic marker OPN was observed in the section corresponding to the hydrogel reinforced with HA, the TNC deposition was more prominent in the anisotropic phase (Figure 11c).

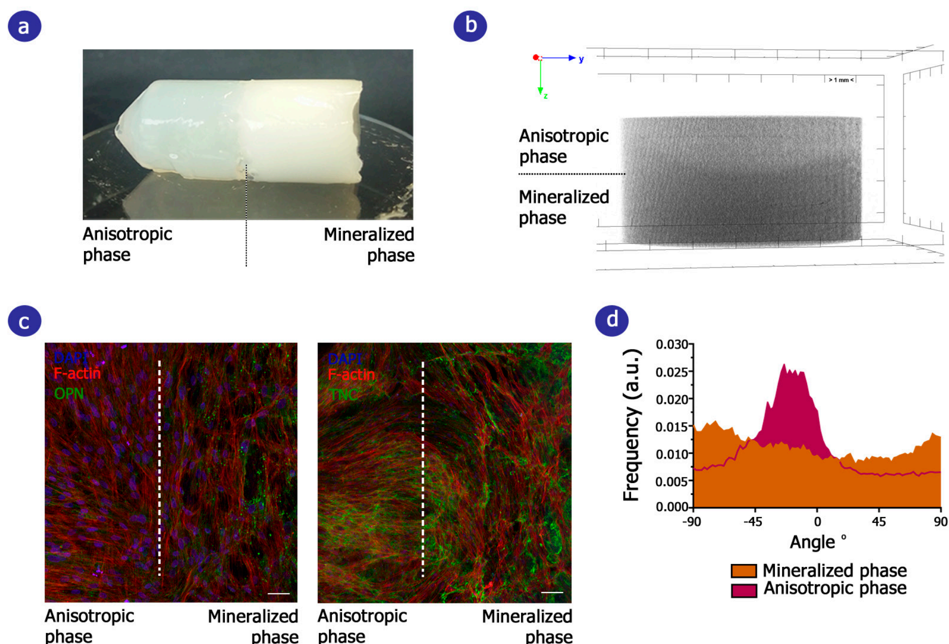

Figure 11. Biphasic hydrogels integrating mineralized and anisotropic features for interfacial tissue engineering. (a) Digital image of produced biphasic hydrogel with well-differentiated phases. (b) 3D reconstruction from micro-computed tomography images of the biphasic hydrogel. (c) Osteogenic differentiation-related marker osteopontin (OPN) and tendon tissue-related marker tenascin (TNC) expression evaluation with hASCs encapsulated in biphasic hydrogels for 21 days. Scale bars = 100 µm. (d) Directionality frequency plots of cytoskeleton alignment analysis in each of the phases.

Together, our data demonstrate that the enzymatic assembly proposed to the development of the biphasic hydrogels allows introduction of compositional gradient without influencing the structural features and biological performance of embedded cells of each phase. Moreover, we envision that the development strategy based on gelatin and the mTG enzyme explored in this work could be further customized to boost the healing process of tendon-to-bone interface tissue. Thereby, taking the advantage of this design versatility, this approach could be derived into multiphase systems by overlaping the tailored hydrogels to add more differential phases. As a proof of concept, we succeeded in preparing a triphasic scaffold with well-differentiated phases.

Complementarily, specific growth factors could be potentially loaded in each of the phases in order to stimulate the stem cells differentiation towards specific phenotype (9). In this regard, further basic research should focus on the effect of particular biochemical factors combination on stem cells differentiation patterns. Furthermore, although the biological assays performed in this work were exclusively done with hASCs, bioinspired heterotypic cellular 3D co-culture platforms could be easily developed by encapsulating different cell types in each of the proposed integrative phases. In this regard, multi-chamber bioreactor devices that are currently under research could be extremely interesting for customizing the culture medium of each phase, thus adapting the appropriate nutrients and conditions for each cell type.

In summary, in the present doctoral thesis we focused on gelatin and the enzymatic crosslinking strategy to develop tailorable 3D platforms aimed at both bone tissue and bone associated interfaces tissue engineering. As a whole, our data represent an important step forward in the consolidation of this biomaterial within the field of regenerative medicine.

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Conclusions

On the basis of the results obtained in the experimental studies of this Doctoral Thesis, the following conclusions were derived:

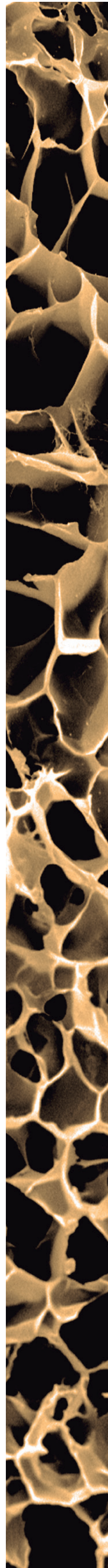
1. Gelatin three-dimensional (3D) scaffolds enzymatically crosslinked with microbial transglutaminase enzyme were successfully developed by the freeze-drying technique. *In vitro* characterization analysis revealed adequate capabilities for both the release of vascular endothelial growth factor (VEGF) and bone morphogenetic protein -2 (BMP-2) growth factors, as well as the support for cell culture.

2. Bioinspired gelatin-based 3D scaffolds reinforced with hydroxyapatite or calcium sulfate stimulated the induction of *in vitro* osteogenic differentiation of human bone-marrow derived mesenchymal stem cells. These composites functionalized by soak-loading with BMP-2 promoted bone regeneration in critical-sized calvarial defects of osteoporotic mice.

3. Gelatin-based hydrogels with anisotropic microstructure were successfully developed by inclusion of cellulose nanocrystals and the exposure of low strength uniform magnetic field during the crosslinking process. These 3D systems induced the aligned growth of encapsulated human adipose tissue-derived mesenchymal stem cells and the oriented deposition of tenascin, glycoprotein related to the extracellular matrix of the tendon tissue.

4. Gelatin-based biphasic hydrogels were developed through the enzymatic assembly of mineralized and anisotropic phases. Gelatin/hydroxyapatite and gelatin/cellulose nanocrystals composites provided tailored functionalities, mimicking bone and tendon tissues, respectively.

EUSKARAZKO BERTSIOA



Sarrera

Gelatina biomateriala Ehun Ingeniaritzarako

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LABURPENA

Ehunen ingeniaritza medikuntza birsortzailearen estrategia terapeutiko garrantzitsuenetariko bat bezala kontsideratu daiteke. Teknologia berri horien helburu nagusia, zauritu edo gaixotutako ehunak eta organoak orbaindu edota birsortzeko gaitasuna duten ordezkoen garapena da. Ordezko hauen bidez giza ehun eta organoek birsortzeko duten ahalmen mugatua gainditu nahi da. Berrikuspen honetan, gelatina materialak ehun ingeniaritzara bideratutako sistema aurreratuak diseinatzeko eta garatzeko berezko dituen propietate egokiak nabarmentzen dira. Gelatina kolagenoaren hidrolisitik eratorritako jatorri naturaleko proteina da. Dokumentu honetan gelatinan oinarritutako sistema konposatuen teknikaren egoera aurkezten da, material polimeriko honen mugak gainditu eta formulazioen ezaugarriak modulatzea helburu izanik. Gelatina konpositeak erabiliz molekula bioaktiboen askapen kontrolatua, eroaleak diren formulazioak edota propietate mekaniko hobetuak dituzten sistemak garatu daitezke. Hainbat ikerketa lanen arabera, kaltzio fosfato gatzak edota hainbat polimero sintetiko ezberdin gelatinarekin konbinatuz gero, propietate mekaniko hobetuak dituzten egiturak garatzea posible da. Bestalde, polianilina edo karbonozko nanosubstratuak, bereziki interesgarriak izan daitezke eroaleak diren gelatinazko sistemak sortzeko garaian, bereziki bihotz eta nerbio-ehunen ingeniaritzaren kasuetan. Azkenik, berrikuspen honek gelatina erabiliz sortu daitezkeen egituren gaineko ikuspegi orokor bat eskaintzen du, hala nola nanopartikulak, mikropartikulak, hiru dimentsiotako (*three dimensional* edo 3D) skaffold-ak, elektroirundako nanozuntzak eta *in situ* gelifikatzen diren formulazioak. Jadanik egin diren aurrerapen garrantzitsuei esker eta etorkizun hurbilean lortzea espero diren beste batzuekin batera, gelatina oinarri duten formulazio aurreratuak klinikara iritsiko direla aurreikusten da.

Hitz-gakoak: biomateriala, gelatina, 3D skaffold-ak, ehunen ingeniaritza, medikuntza birsortzailea, partikulak, hezurra.

1. SARRERA

Teknologia eta biomaterialen bidezko estrategia terapeutiko berrien behar klinikoa gero eta handiagoa da, zauritu edo gaixotutako ehunak eta organoak sendatu, konpondu edo birsortzeko. Nahiz eta eskaera hori ehun mota guztietarako den handia, bereziki premiazkoa da muskulu-eskeletiko sistemako arazo kroniko eta akutuetan: hezur, kartilago, tendoi edo lotailuen traumatismoak eta baita ornoarteko diskoen endekapen edo artrosi kasuetan ere. Ameriketako Estatu Batuetan soilik, urtero 34 milioi prozedura kirurgiko burutzen dira patologia hauen tratamendu modura [1].

Egungo organo emaileen eskasiak eta organo transplanteekin lotutako inmunitate arazo larriek, zientzia alor berrien garapena bultzatu dituzte, hala nola ehunen ingeniaritza [2] eta medikuntza birsortzailea. Azken hau, kontzeptu biologiko eta ingeniaritzarekin erlazionaturiko printzipioak biltzen dituen alor modura definitu daiteke, non zauritutako ehun eta organoen birsortze prozesua bultzatuko duten teknologien garapena duen helburu [3]. Gai honetan jarduteko hainbat estrategia ezberdin garatu izan dira. Bereziki interesgarria kontsideratu daiteke biomaterialez osatutako skaffold-en fabrikazioa, zeintzuek ehun naturala aldi baterako ordezkatuko duten, birsortze prozesua bultzatzatzen duten heinean. Matrize hauek aldi baterako euskarri estrazelular artifizialak dira eta ehun ingeniaritzako osagarri nagusi direla kontsideratzen da, zelula eta faktore biologikoekin batera. Hori dela eta, inplantatu daitezkeen egitura porotsu horiek zelulen eramaile eta printzipio aktiboen gordailu edota bi funtzioak uztartzen dituen eramaileak izan daitezke. Gainera, hainbat dira matrize bat ideal egingo duten ezaugarriak. Batetik, biobateragarriak izan behar dute, erantzun inmunologiko eta hanturazko prozesu minimoa eraginez. Bestetik, biodegradagarriak izateaz gain, euren degradazio tasak birsortze prozesuarekin bat etorri behar du eta gainera porotasun handia erakutsi behar dute. Zelulen askapenerako sitemen kasuan, poroen presentzia beharrezkoa izango da zelulen atxikipena, barneratzea, proliferazioa eta matrize extrazelularraren (*extracellular matrix* edo ECM) deposizioa modu eraginkorrean gertatu dadin. Skaffold-aren funtzioa, zelulen hazkuntza bideratzea izango da, bai sistema barnean ereindako zelulena eta baita inguruko ehunetatik migratutako zelulena ere. Horretaz gain, zelula dentsitate altuak erein ahal izateko poroen arteko konektibitatea handia izatea nahitaezkoa izango da, izan ere nutriente eta degradazio produktuen elkartrukea baimenduko dute inguruarekin. Propietate mekanikoei dagokienez, jatorrizko ehunaren ezaugarri antzekoak izan beharko dituzte eta zelulak tentsio indarretatik babestu. Gainazaleko egitura kimikoak eta topografiak ere funtsezko zeregina dute zelulen arteko elkarrekintza eta ehunen garapen prozesuetan [4].

Jakina da biomaterialek eta material hauetan oinarritutako skaffold-ek funtsezko rola jokatzen dutela medikuntza birsortzaile modernoan. Izan ere, material hauek zelulen funtzioaren gidari izan daitezkeela ikusi da [2]. Azken urteetan, biomaterial berritzaileen diseinu

eta fabrikaziorako irizpideetan aldaketa nabarmena gertatu izan da, biologiaren, ingeniaritza biomedikoaren eta farmazia teknologiaren printzipioak ustartzea lortu baita.

Gelatinak propietate biologiko eta teknologiko egokiak dituenez alor mediko, farmazeutiko eta kosmetikoan oso erabilia izan den biopolimero naturala da. Aplikazio farmazeutiko eta medikoetan, gelatina matrize inplantagarrien osagai moduan, formulazio injektagarrietan, txerto askoren fabrikazioan egonkortzaile gisa, kapsula bigun eta gogorrak egiteko garaian, plasma hedatzaile gisa eta baita zaurien zainketa eta tapoi hemostatiko moduan ere erabili izan da [6-8]. Horretaz gain, azken urteetan aurrerapauso handiak egin dira gelatina ehun ingeniaritzako egituren diseinuetan biomaterial modura erabili ahal izateko.

Berrikuspen honetan, ehun ingeniaritzako biomaterial gisa gelatinak duen zeregin nagusia nabarmentzen da, ehunen konponketa eta birsorkuntza prozesuan eskaintzen dituen abantailak goraipatuz. Gainera medikuntza birsortzailearen estrategietan erabiltzeko proposatu diren gelatina konpositeen ikuspegi orokor bat biltzen da. Hori dela eta, gelatinan oinarritutako formulazioen aplikazio kliniko potentziala eztabaidatzen da, mikro eta nanopartikulak, 3D matrizeak eta *in situ* gelifikatutako produktuak barne.

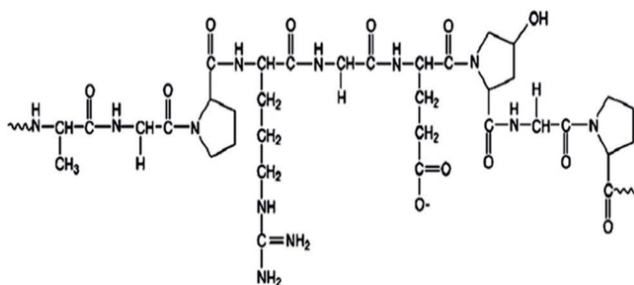
2. GELATINAREN EZAUGARRIAK

Gelatina jatorri naturaleko proteina da, zeina I motako kolagenoaren hidrolisi kimiko, fisiko edota entzimatikotik eratorria den. I motako kolagenoa animalien (arrain, ugaztun eta hegazti) azal, hezur eta ehun konektiboaren osagai nagusiena da. Elkar konektatuta dauden proteina kateek osatzen dute kolagenoa. Aipatutako prozesu hidrolitikoan, kolagenoaren bereizgarri den helize hirukoitz egitura apurtu egiten da eta harizpi bakarreko makromolekulak lortzen dira.

2.1 Gelatinaren egitura

Gelatinaren egitura primarioa proportzio aldakorrean agertzen diren hogei aminoazido baino gehiagok osatzen dute (1. Irudia). Jatorriaren arabera, aminoazido konposizioa eta berauen sekuentzia ezberdina izan daiteke, gelatinaren ezaugarrietan eragin zuzena dutelarik. Gelatina glizina-X-Y peptidoen errepikapenez osatuta dagoela kontsideratu daiteke, non teorikoki X eta Y posizioan edozein aminoazido agertu daitezkeen arren, ohikoena X posizioan prolina eta Y posizioetan hidroxiprolina agertzea da. Gelatina, lotura peptidikoen bitartez mihiztatutako aminoazido ezberdin nahaste gisa kontsideratu daiteke, 15-400 kD bitarteko pisu molekularreko polimeroak osatzen dituztelarik. Gelatina lortzeko prozesuan erabilitako parametro espezifikoek (tenperatura, pH, denbora), aukeratutako aurretratamendu metodoak eta erabilitako oinarrizko materialaren ezaugarriek lortutako polipeptido kateen luzeran eragina izango dute. Izan ere, prozesu hidrolitikoan zehar, kolagenoaren

helize hirukoitza osatzen duten hiru kate polipeptidikoak hautsi egiten dira eta polimero kate bakarra (α katea), kobalenteki lotutako bi kate (β-kateak) edo elkarren artean lotutako hiru kate (γ-kateak) lortzen dira. Lortutako kate mota bakoitzaren proportzioa ezberdina izaten da aurrez aipatutako aldagaien arabera, aminoazido kateen pisu molekularra ezberdina izanez. Gelatina molekulen artean elkarrekintza ez-kobalenteak ezartzen dira, hala nola hidrogeno zubiak, van der Wall indarrak eta elkarrekintza elektrostatiko eta hidrofobikoak [10]. Beraz hainbat gelatina mota daudela esan daiteke, erabiltzen den kolagenoaren iturria eta burututako tratamendu hidrolitikoaren arabera ezberdintzen direlarik. Gelatina lortzeko prozesu osoa hiru fase ezberdinetan banatu daiteke: Kolagenoaren isolamendua eta aurretratamendua, gelatinaren erauzketa eta purifikazioa eta lehortze fasea [3].



1. Irudia. Gelatinaren egitura kimikoa. [4] erreferentziatik, baimenarekin erreproduzitua.

2.2 Gelatina lortzeko iturriak

Hainbat gelatina mota lortu daitezke iturriaren arabera. Ugaztunetatik eratorritako gelatinak izan dira birsorkuntza helbururetarako gehien erabili direnak. Nagusiki, gelatina mota hauek txerri eta behi animalietatik lortzen dira, bereziki azal eta hezurretatik hain zuzen ere [12]. Hala ere, esan beharra dago material hauek ez direla ehun ingeniaritza alorrean erabiltzeko biomaterial idealaren prototipo egokienak, osasun segurtasun eta hainbat aspektu sozio-kulturak direla medio. Izan ere, kontuan izan behar da gaixotasunen transmizioa gertatzeko aukerak egon daitezkeela, esaterako prioien transmizioa eta entzefalopatia espongiformearen garapen arriskua. Haatik, zenbait ikerketek frogatu dutenez, ugaztunen kolagenotik gelatina lortzeko prozesua bera metodo eraginkorra da lehengaian egon litezkeen prioiak ezabatzeko [11]. Alabaina, azken hamarkadan beste hainbat iturritako gelatinak erauzi dira, hala nola arrain, hegazti eta ornodunetatik, ugaztunetatik eratorritako gelatinen alternatiba moduan. Esan beharra dago, gaur egun hegaztietatik eratorritako gelatinen produkzio komertziala oraindik mugatua dela, legearekin erlazionatutako baldintzak direla medio. Hori dela eta, badirudi arrainetatik eratorritako gelatina alternatiba hobea izan daitekeela. Ur epel eta ur hotzetako arrainen azal, hezur eta hegaletatik eratorritako gelatinak hainbat metodo ezberdin erabiliaz, 1960 urteaz geroztik ekoiztu izan dira [13-17]. Arrainetatik eratorritako gelatinak, txerri jatorriko gelatinen ezaugarri antzekoak badituzte

ere, aminoazidoen sekuentzia eta proportzioak ezberdinak dira. Izan ere, arrain jatorriko gelatinen prolina eta hidroxiprolina aminoazidoen edukia txikiagoa da, eta aminoazido hauek ordenatutako egitura finkatzen laguntzen dute, gel sarea osatzeko garaian. Horregatik, ezberdinak dira arrain eta txerri jatorriko gelatinen hainbat eta hainbat ezaugarri: urtze tenperatura, gelifikazio tenperatura, gel modulua, egonkortasun termikoa edota biskositatea. Aipatutako jatorriko bi gelatinak alderatuz gero, arrain jatorrikoek urtze tenperatura baxuagoa, egonkortasun termiko txikiagoa eta biskositate altuagoa izaten dute [18].

Ehunetatik eratorritako gelatinen desabantailak gainditu eta materialen propietateak hobetzeko helburuarekin, berriki gelatina birkonbinatuak garatu dira. Teknologia honekin pisu molekular eta puntu isoelektriko zehatzeko gelatinak prestatzea posible da. Gainera, prestatutako loteen arteko erreproduzigarritasuna handiagoa izatea lortu da. Bi estrategia ezberdin garatu dira giza gelatina errekonbinantea ekoizteko. Aukera batean lehendabizi kolageno errekonbinantea sortzen da, ondoren purifikatu eta azkenik desnaturalizazioa eragin (kateen zatiketa burutuaz edo ez), amaierako produktu gisa gelatina errekonbinantea lortzen delarik. Zuzenean aurrez definitutako α-kate espezifikoak ekoiztea da beste aukera. Proteina errekonbinante hauen espresioa burutzeko hainbat sistema ezberdin erabili izan dira, hala nola *Pichia pastoris* edota *Hansenula polymorpha* legamiak, *Escherichia coli* bakterioa, tabakoaren landarea eta baita saguak ere [19].

2.3 Propietate biologikoak

Gelatina lortzeko iturriaren arabera, gelatinen hainbat propietate ezberdinak badira ere, gelatina guztietan berdinak diren hainbat ezaugarri antzeman dira. Gelatina, oro har material biobateragarria dela frogatu da, ez zitotoxikoa eta immunogenikotasun baxua erakutsi du kolagenoarekin alderatuz gero. Estatu Batuetako farmako eta elikagaien administrazio (*Food and Drug Administration* edo FDA) erakundeak gelatina material segurua dela kontsideratu du, orokorrean seguru bezala onartua (*Generally Regarded As Safe* edo GRAS) maila emanez [20]. Horretaz gain, gelatinaren egituran biologikoki funtzionalak diren domeinuak aurkitu daitezke, hala nola arginina-glizina-azido aspartiko (*arginine-glycine-aspartic acid* edo RGD) sekuentzia. Sekuentzia espezifiko honek zelulen atxikipena, desberdintzapena eta proliferazioa hobetzen ditu [21]. Bestalde, bere egituran biodegradagarri egiten duten eremuak daude. Sekuentzia espezifiko hauek matrizearen proteinasa metalikoak (*matrix metalloproteinases* edo MMP) ezagutu eta entzimatikoki degradatzeko aukera eskaintzen dute. Gainera, degradazio entzimatikoaz sortutako azpiproduktu horiek ere biobateragarriak direla ikusi da [22].

2.4 Propietate fisiko-kimikoak

Gelatinaren propietate fisiko-kimikoei dagokienez, uretan disolbagarria den polipeptidoa dela esan behar da eta portaera anfoterikoa erakusten du aminoazido alkalino eta talde

funtzional azidoak baititu [23]. Hala ere, bere karga elestrostatikoa erauzketa egiteko erabiltzen den tratamenduaren araberakoa izango da, puntu isoelektriko ezberdineko gelatinak lortzen direlarik.

2.4.1 Puntu isoelektrikoa

Erauzketa prozesuaren aurretik kolagenoaren tratamendua egiteko erabiltzen den metodoaren arabera, nagusiki bi gelatina mota ezberdintzen dira: A mota eta B mota. A motako gelatinaren puntu isoelektrikoa 9.0-koa izaten da eta bere erauzketa kolagenoaren hidrolisi azidoa eraginez lortzen da, azido sulfurikoa edota klorhidrikoa erabiliz. Aldiz, tratamendu alkalinoa erabiliz gero, 5.0-ko puntu isoelektrikodun B motako gelatina lortzen da. Kasu honetan, asparagina eta glutaminaren amida taldeak hidrolizatzen dira, karboxilo taldedun aspartatoa eta glutamato hondarrak sortuz [24].

2.4.2 Propietate termo-itzulkorra

Gelatinaren propietate esanguratsuenetako bat gel termo-itzulkorrak osatzeko gaitasuna da, gelatina soluzioa hozterakoan gertatzen den konformazio-transizioa dela eta. Gelatina granuluak uretan barneratzean, puztutako partikulak eratzen dituzte. Partikula hauek berotuz gero, desegin egiten dira gelatinazko disoluzioa lortuaz. Gelifikazio prozesuan zehar, tokian-tokiko eremu ordenatuak eratzen dira, zeintzuak lotura ez-espezifiko bitartez batu egiten diren [25]. Molekula arteko elkarrekintza hauek orokorrean hidrofobikoak, hidrogeno loturak edota lotura eletktrostatikoak izaten dira. Polimerozko gel termo-itzulkorra, tenperatura jakin batean biskositatea erabat aldatzen den polimero kateen elkargurutzaketa ondorioz sortzen den gel moduan definitu daiteke [26]. Gelatinaren kasuan, elkagurutzaketaren lotura-energia nahiko ahula denez, gel fisikoak termikoki itzulgarriak dira [25].

Gelatina disoluzioaren portaera hainbat faktoreren menpekoa da: tenperatura, pH-a, prestatzeko erabilitako metodoa, historia termikoa eta kontzentrazioa. Gelatina disoluzioa 40 °C-tik gora berotzean, bere erresistentzia eta biskositatea txikiagotu egiten dira. Hala ere, eratutako gelaren zurruntasuna ez da tenperaturaren menpekoa soilik. Izan ere, kontzentrazioak, berezko indarrak (bloom indarra), pH-ak eta bestelako gehigarrien presentziak ere gelaren zurruntasuna aldatu dezakete [27]. Bloom indarrak hozterakoan sortzen den gel fisikoaren indarrari egiten dio erreferentzia [28]. Bloom indarraren balioa gelatinaren egitura eta masa molekularraren araberakoa izaten da.

2.4.3 Gelatinaren elkargurutzaketa

Tenperatura fisiologikoan egonkotasun termikorik ez izatea da ehun ingeniaritzako aplikazioetan gelatina erabiltzearen eragozpen nagusiena. Hori dela eta, elkargurutzaketa bideratuko duen osagaien erabilera ezinbesteko bihurtzen da gelatina biomaterial modura erabiltzeko. Gelatina kateen elkargurutzaketa burutuaz, alde batetik egonkortasun termiko eta mekanikoa hobetzen dira baldintza fisiologikoetan, eta bestalde *in vivo* gertatutako

degradazio tasa murrizten da [29]. Gelatinaren erretikulazioa gauzatzeko erabilitako osagai motaren arabera, sortutako hidrogelaren propietate fisiko, mekaniko eta zitotoxikoak ezberdinak izango dira. Elkargurutzaketa burutzeko erabilitako metodoak bi multzo nagusitan sailkatu daitezke: gurutzaketa fisikoa eta gurutzaketa kimikoa. Gurutzaketa fisikoa eragiteko orduan ez dago toxikoa izan daitekeen edozein agente kimiko erabiltzeko beharrik, ezta gelatinan aldaketak egitekoa ere. Kasu hauetan baina, zaila gertatzen da erretikulazioaren dentsitatea kontrolatzea eta sarritan, prozesuaren eraginkortasuna txikia izaten da [30]. Mikrouhinen energia [31], tratamendu dehidrotermala [32-34] edota erradiazio ultramorea [35] erabili izan dira, fisikoki elkargurutzatutako gelatina hidrogelak lortzeko. Bestalde, gurutzaketa kimikoan, konposatu kimikoak erabiliz gelatina kateen erretikulazioa lortzen da. Bi-funtzionalak edota polifuntzionalak izan daitezkeen konposatuak gelatina molekula artean lotura kimikoak osatuz txertatzen dira. Lotura kimiko horiek, sarritan lisina eta hidroxilisinaren amino talde askeekin edo glutamato eta aspartato aminoazidoen karboxilo taldeekin osatzen dira [30]. Gurutzaketa kimikoa duten gelatina hidrogelen degradazio prozesuan, erreaktiboak edo toxikoak izan daitezkeen produktuak askatzeko aukera egon daiteke, zeintzuak gorputzean kalteak eragin ditzaketen [36]. Prozesu hau jarraitzen duten lotura eragileen adibideak aldehidoak (glutraladehidoa [37] edo glizeraldehidoa [38]), poliepoxidoak eta isozianatoak dira [39]. Konposatu hauek ez-zero luzerako erretikulatzaile moduan izendatzen dira. Beste konposatu mota batzuk, aldiz, gelatina molekularen karboxilo taldeak aktibatu eta inguruko proteina katearen aminoazidoekin erreakzionarazten dituzte, molekula barneko amida loturak sortuz. Kasu hauetan, zero-luzerako erretikulatzaile bezala ezagutzen diren konposatuak ez dira azken egituran barneratzen [30].

Tradizionalki, aldehidoak izan dira gelatina erretikulatzeko gehien erabili diren osagaiak. Hala ere, konposatu hauek ez dira zelulak kapsularatzeko erreaktibo egokienak, euren degradazioaren ondorioz sortutako produktuek sorrarazitako zitotoxikotasuna, immunogenizitatea eta hantura erantzuna dela eta. Horregatik, gero eta interes handiagoa gailendu da efektu zitotoxiko txikiagoa duten gelatina erretikulatzaile naturalengan. Adibidez, genipina konposatua *Gardenia jasmoides* landarearen loreetatik erauzitako erretikulatzaile naturala da. Konposatu hau aplikazio biomedikoetara bideratutako materialak elkargurutzatzeko erabili izan da. Konposatu kimiko honek zitotoxikotasun baxua, elkargurutzaketarako efizientzi altua eta biobateragarritasun ezaugarri onak erakutsi ditu [40,41].

Bestalde, ehunen ingeniaritzarako entzimatikoki erretikulatutako hidrogelak garatzen ari dira azken urteotan. Izan ere, erreakzio hauek baldintza fisiologikoetan gertatzen dira eta sustratuarekiko espezifikotasuna lortu daiteke. Proposatu diren erreakzio entzimatiko gehienak pH neutroan katalizatzen dira, ingurune urtsuan eta tenperatura moderatuetan [42]. Orokorrean, tirosinasak, transferasak edota peroxidasa entzima motak erabiliz prestatu izan dira hidrogelak [43]. Gelatinaren kasuan, transglutaminasa mikrobiarra, [45,45] eta

perretxikoetatik eratorritako tirosinasa [46,47] erabiliz elkargurutzaketa eraginkorra lortu izan da formulazio ezberdinen garapena baimenduaz.

Gaur egun arte, gelatinaren elkargurutzaketa aurrez bere egituran aldaketarik egin gabe burutu izan da. Hala ere, gelatinaren egituran aldaketak eraginez gero elkargurutzaketa prozesuaren kontrola handiagotu daitekeela ikusi da. Kasu hauetan, oro har, gelatinaren egituran talde funtzionalak txertatzen dira eta amaierako hidrogelaren diseinu eta propietateen gaineko kontrola handitzea lortzen da. Gelatina aldatzeko hainbat talde funtzional ezberdin erabiltzen hasi dira, hala nola akrilamidak, norborenoa, metakrilo taldea edota azido felurikoa [28]. Zentzu honetan, badirudi metakrilatutako gelatinak biltzen dituela aplikazio biomedikoetarako ezaugarri egokienak. Izan ere, gelatina egituran burututako aldaketa honek ez dio eragiten RGD sekuentziari, ezta MMP sekuentzia sentikorrei ere [36,48].

3. GELATINAN OINARRITUTAKO SISTEMA KONPOSATUAK

Aurretik aipatutako moduan, gelatinak hainbat ezaugarri egoki aurkezten ditu ehunen ingeniaritzan erabili ahal izateko. Hala ere, gelatina oinarri duten sistema konposatuak garatzeko ahaleginak egiten ari dira, biomaterial honek izan ditzakeen hutsuneak betetzeko asmoarekin. Helburua, propietate ezberdinak dituzten materialen arteko sinergiak bilatzea izan da, ehun ingeniaritzaren eskakizun partikularrei erantzuna emateko propietate hobeak dituzten formulazio konposatuak lortuaz. Hori dela eta, azken urteotan, bi material edo gehiago uztartuta dituzten formulazio aurrerakoiak garatu izan dira [20].

Zentzun honetan, sistema konpositeen garapenarekin lortutako helburuak, besteak beste, gelatina formulazioen propietate mekanikoen hobekuntza, eroaleak diren formulazio berriak garatzea eta molekula bioaktiboen askapena kontrolatzeko sistemak diseinatzea izan dira (1.Taula).

3.1 Propietate mekanikoen hobekuntza

Hidrogelen ur-eduki handia dela eta, euren indar mekanikoa mugatua izaten da oro har. Ezaugarri bereizgarri horrek zaildu egiten du berauen erabilera baldintza mekaniko handiak behar diren egoeretan, hala nola hezur [49] edo kartilago [50,51] kasuetarako. Gelatinaren kasuan [52], ezaugarri hauek erretikulazioa burutuaz handitzen badira ere, sarritan ez da lortzen beharrezko eskakizunak betetzea [53]. Azken urteotan hainbat saiakera egin dira muga horiek gainditzeko helburuarekin. Horietako bat, formulazioan bigarren material bat integratzea izan da, material konposatu hibrido bat sortuz [54].

Hezur-ehuna adibidez, bere horretan konposite natural bat dela kontsideratu daiteke, fase organiko eta fase mineral baten konbinazioz osatuta dagoelarik. Fase organikoa, batez ere I motako kolagenoan da aberatsa eta fase minerala, aldiz, funtsean kaltzio fosfato kristalez osatuta dago, batez ere hidroxiapatita (*hydroxyapatite* edo HA) [55].

1.Taula: Gelatinan oinarritutako sistema konposatuen adibideen laburpena

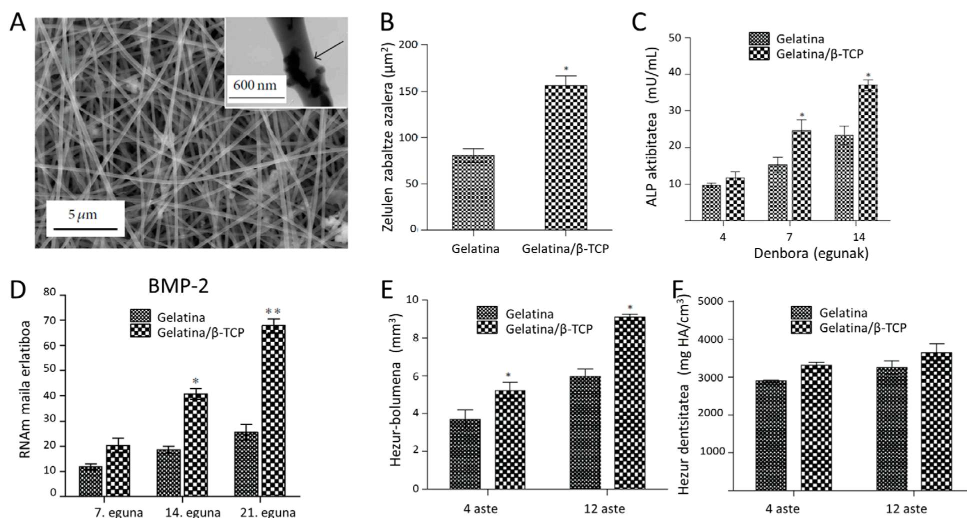
Hobetutako propietatea	Konposatuaren materialak	Formulazio mota	Erabilera	Erref.
Propietate eroaleak	Gelatina/PANI	Nanozuntz skaffold-a	Bihotz ehunaren ingeniaritza	[109]
	Gelatin/kitosanoa/PANI/grafenoa	Nanopartikula eroaledun skaffold-a	Nerbio periferikoen birsorkuntza	[110]
	Gelatina/horma anitzeko CNT	Nanozuntz skaffold-a	Muskulu eskeletikoen ehun ingeniaritza	[127]
	Poliglizerol-sebazatoa/gelatina/CNT	Nanozuntz skaffold-a	Bihotz ehunaren ingeniaritza	[128]
	Gelatina metakrilatua/GO	Hidrogel injektagarria	Miokardioko infartu akutuaren terapia	[121]
	Gelatina metakrilatua/ GO erreduzitua	3D skaffold-a	Bihotz ehunaren ingeniaritza	[122]
	Gelatina/HA/GO	3D skaffold-a	Hezurren birsortzea	[123]
Biomolekulen askapenerako kontrola	Gelatina/hialuronatoa/heparina	bFGF, VEGF, Ang-1, KGF, PDGF eta TGF-β dituen hidrogel film-a	Angiogenia ehunen ordezkoetan	[134]
	Gelatina/PVA/kitosanoa	Gelatin nanopatikuladun nanozuntz sarea	Aktibitate antimikrobiarra zaurietan	[135]
	Gelatina/zeta/kitosanoa/glizerofosfatoa	PDGF-z kargatutako gelatinazko mikroesferadun hidrogel injektagarria	Kartilago ehun-ingeniaritza	[136]
	Gelatina/OPF	TGF-β eta IGF-1-z kargatutako gelatinazko mikropartikuladun hidrogela	Ehun osteokondralaren birsorkuntza	[138]
	Gelatin/PLGA/PPF	BMP-2-z kargatutako PLGA mikroesferadun PPF/gelatin hidrogela	Hezur ehun-ingeniaritza	[139]
Propietate mekanikoak	Gelatina/kitosanoa/alginatoa/HA	3D skaffold-a	Hezur ehun-ingeniaritza	[63]
	Gelatina/nanoHA	3D skaffold nanoegituratua	Hezur ehun-ingeniaritza	[64,66-68]
	Gelatina/TCP	Lumbrokinasa-dun 3D skaffold-a	Hezur ehun-ingeniaritza	[70,72,74]
	Gelatina/TCP	Nanozuntz mintza	Hezur ehun-ingeniaritza	[71]
	Gelatina/PVA/BCP	Elektroirundako nanozuntzen sarea	Hezur ehun-ingeniaritza	[75]
	Gelatina/pektina/BCP	BMP-2 eta VEGF-duen 3D skaffold-a	Hezur ehun-ingeniaritza	[76]
	Gelatina/azido hialuronikoa /BCP	3D skaffold-a	Hezur ehun-ingeniaritza	[78]
	Gelatina/OCP	3D skaffold-a	Hezur ehun-ingeniaritza	[79-81]
	Gelatina/alginatoa/BCP/HA	3D skaffold-a	Hezur ehun-ingeniaritza	[82]
	Gelatina/PLA	Nanozuntz skaffold-a	Hezur ehun-ingeniaritza	[87,88,90]
	Gelatina/PLA	Nanozuntzezko skaffold tubularra	Odol-hodien ehun ingeniaritza	[89]
	Gelatina/PCL	Zuntzezko matrizea	Odol-hodien ehun ingeniaritza	[35]
	Gelatina/PCL/hezur hautsa	Elektroirundako zuntzen sarea	Hezur ehun-ingeniaritza	[93]

PANI – polianilina, CNT – karbonozko nanohodiak, GO – grafeno oxidoa, HA – hidroxiapatita, PVA – Alkohol polibinilikoa, OPF- oligopolietilenglikol fumaratoa, PLGA – azido poli(laktiko-ko-glikolikoa), TCP – trikaltzio fosfatoa, BCP – kaltzio fosfato bifasikoa, OCP – Oktakaltzio fosfatoa, PLA – azido polilaktikoa, PCL – polikaprolaktona, bFGF – fibroblastoen hazkuntza faktore b, VEGF – endotelio baskularraren hazkuntza faktorea, Ang-1 – angiopoietina 1, KGF – keratinozitoen hazkuntza faktorea, PDGF – plaketatik eratorritako hazkuntza faktorea, TGF-β – hazkuntza faktore eraldatzaile β1, IGF-1 – intsulinaren antzeko hazkuntza faktorea -1, BMP-2 – hezur-proteina morfogenikoa -2

Kasu honetan, kaltzio fosfatoa hezurraren konpresio indarraren arduraduna da eta kolagenoak ezaugarri elastikoak ematen dizkio, aldiberean gogorra eta elastikoa den ehuna lortuz [56]. Hori dela eta, gelatinaren propietate mekanikoak hobetzen dituzten konbinazioak bereziki interesgarriak suertatzen dira hezur-birsorkuntzara zuzendutako formulazioen garapenean, hezur-ehunaren mimetikoa izango den sistema eraikitzeko. Skaffold-ak aurkeztuko dituen propietate mekanikoak (modulu elastikoa, trakzio indarra, haustura gogortasuna, luzapen portzentaia), berau fabrikatzeko erabilitako materialaren araberakoak izango dira. Propietate hauek, ordezkatu edo birsortu nahi den hezurraren antzekoak izatea gomendatzen da. Izan ere, hezur injertuekin lotutako lesioetako batzuk osteopenia edota hezur-galerak izaten dira. Kontuan izan behar da, hezur kortikalak eta hezur esponjotsuek ez dituztela ezaugarri mekaniko berdinak. Horrenbestez, ez da erraza ezaugarri guztiak antzekoak izango dituen skaffold ideal bat diseinatzea [57]. Berriki, hainbat sistema konposatu diseinatu eta fabrikatu dira, gelatina zeramikekin, jatorri naturaleko polimeroekin edota polimero sintetikoekin konbinatuz, baita material horien nahasketak prestatuaz ere [58].

3.1.1 Kaltzio fosfatozko zeramikak

Kaltzio fosfatozko zeramikak kaltzio ioiez eta ortofosfato, metafosfato edo pirofosfatoez osatutako material bioaktiboak dira. Konposatu hauek oso interesgarriak dira hezur-ehunaren ingeniaritzan. Izan ere, polimeroz osatutako skaffold-en propietate mekanikoak areagotzen dituzte eta gainera, horietako askok ezaugarri osteokonduktoreak dituzte (osteoblastoak atxikitzea eta proliferatzea bultzatzen dute) eta hainbatek ezaugarri osteoinduzitzaileak ere aurkezten dituzte (hezur berriaren eraketa estimulatzen dute zelula progenitoreen biltzea sustatuz eta osteoblastoekin erlazionatutako zeluletara ezberdintzatzea bultzatuz). Konposatu zeramiko hauen solubilitate propietateek, kristalinitateak eta kaltzio eta fosfato kantitate ratioak eragiten dituzte osifikazioa bultzatzeko dituzten gaitasunen ezberdintasunak [59-61]. Badirudi, hezur-ehunen ingeniaritzako ordezko ezin hobeak bildu beharreko ezaugarri garrantzitsuenei dagokienez, osteoindukzioa eta osteokondukzioa bideratzeko gaitasuna eta birsortu edo ordezkatu beharreko hezurraren modulu elastiko eta trakzio indar antzekoak izatea direla [62]. Kaltzio fosfatozko zeramikak gelatinarekin batera konbinatu dira, hezur-ehunen ingeniaritzan erabiltzeko beharrezko propietate guztiak betetzen dituen sistema sortzeko helburuarekin (2. Irudia). Konposatu horien artean HA, trikaltzio fosfatoa (t*ricalcium phosphate* edo TCP) [70-74], kaltzio fosfato bifasikoa (*biphasic calcium phosphate* edo BCP) [75-78] eta oktakaltzio fosfatoa (*octacalcium phosphate* edo OCP) [79-81] aurkitzen dira. Zenbait kasutan, formulazio berean hainbat kaltzio fosfato mota ezberdin konbinatu izan dira sistema hobeak lortzeko helburuarekin [82].



2. Irudia. Hezur-muineko zelul-ama mesenkimalen (*bone-marrow mesenchymal cells* edo BM-MSC) ezberdintzatze osteogenikoa eta *in vivo* hezurraren formazioa handitzeko, elektroirutez lortutako gelatina/β-TCP nanozuntzez osatutako sistema konposatua. (A) Elkargurutzatutako nanozuntzen SEM eta TEM irudiak. (B) BM-MSC zelulen hedadura azalera eta (C) ALP aktibitatea denboran zehar. (D) BMP-2 gene osteogenikoaren RNAm-aren espresio maila. Hezur-bolumena (E) eta hezur-dentsitatearen (F) azterketa kuantitatiboa, skaffold-ak arratoien garezurrean inplantatu eta 4 eta 12 asteetara. (* $p<0.05$). [71] erreferentziatik moldatua.

Hezur-ehunen ingeniaritzan gehien erabiltzen diren kaltzio fosfatozko zeramiketako bat da HA, hezur matrizearen osagai ez-organikoan dagoen kaltzio fosfato kristala ($Ca_{10}(PO^4)_6(OH)_2$) hain zuzen ere. Hezur-ehunen ingeniaritzarako aztertutako konposite garrantzitsuenetariko bat HA eta gelatinaren arteko nahastea izan da [83]. Badaude alde batetik, soilik gelatina eta HA dituzten formualazioak eta bestalde, polimero mota ezberdinen konbinazioak HArekin uztartzen dituzten sistema aurreratuak ere [63].

Adibide gisa, osteoi biomimetikoa garatzeko asmoz, HA eta gelatina proportzio ezberdinetan konbinatu izan dira. Zurruntasun mekaniko handiagoa eta biobateragarritasun hobea zuten hidrogela prestatu eta bi eraztun kontzentriko zituen egitura batean bildu ziren. Barneko eraztunean giza zilbor-zaineko zelula endotelialak (*human umbilical vascular endothelial cells* edo HUVEC) erein ziren odol-hodiak sorrarazteko eta, kanpoko eraztunean aldiz MG63 osteoblasto zelulak kapsularatu zituzten [65].

Beste alde batetik, BCP zeramikak solubilitate ezberdineko bi fase dituzten zeramikak dira. Disolbagarritasun txikiko apatita fasea alde batetik, eta solugarriagoa den osteoindukziorako gaitasuna duen fasea [84]. Nanopartikulak prestatzea konposatu hau beste material batzuekin konbinatzeko estrategia interesgarria da. Hezurraren birsorkuntzarako

erresistentzia mekaniko egokidun nanozuntzez osatutako skaffold-a sortzeko, BCP nanopartikulek egonkortasuna areagotzea lortu zuten. Garrantzitsua da aipatzea, formulazio berri horrek ezaugarri mekanikoak hobetzeaz gain, propietate biologikoak ere hobetu zituela. Osteoblastoen atxikipena eta osteopontina proteina osteogenikoaren espresioa handiagoa izan zen, zeramikarik ez zeukan skaffold-ekin alderatuz gero. Skaffold-en ezaugarrien hobekuntza hori ez zen soilik *in vitro* ikerketetan antzeman, baita arratoien garezur-gangako animalia ereduan ere, non 2 eta 4 asteetan hezurraren formazioa handitu egin zela antzeman zen [75].

Kaltzio fosfatozko zeramikak erabiltzeko garaian materialek aurkezten duten biodegradazioa kontuan hartu behar da. Hain zuzen ere, zeramika hauen birxurgapena azaltzeko hainbat mekanismo proposatu izan dira [57]. Chiba S. *et al.*-ek kaltzio fosfato materialen birxurgapen tasa eta osteokonduzio prozesua aztertu zituzten. Lau material ezberdin erabili ziren birxurgapen tasa eta birsortutako hezurraren kalitatea ebaluatzeko. OCP/gelatina sistema konposatuaren kasuan birxurgapen osoa gertatu zela antzeman zuten arren, TCPz osatutako inplante komertzialak ez ziren guztiz birxurgatu [79].

3.1.2 Polimero sintetikoak

Sintetikoak diren polimero ezberdinak ehunen ingeniaritzan maiz erabili izan dira helburu ezberdinetarako. Propietate mekaniko oso ezberdinak dituzten material multzo handi bat osatzen badute ere, guztiak baldintza kontrolatuetan sintetizatu ahal dira, haien propietate mekaniko eta fisikoak aurresan daitezke eta erreproduzigarriak dira. Hala ere, bioaktibitate mugatua aurkezten dute, sarritan gainazal hidrofobikoa izaten baitute [85]. Hori dela eta, gelatina bere ezaugarrien osagarriak izango dituzten polimero sintetikoekin konbinatzea oso interesgarria suertatzen da, ordezkatu nahi den ehunaren baldintzak beteko dituzten sistemak diseinatzeko. Alde batetik, polimero sintetikoek funtzio estrukturala eskainiko dute eta gelatinak, bestalde, propietate biologikoz hornituko du skaffold-a [86].

Azido polilaktikoa (*polylactic acid* edo PLA) eta polikaprolaktona (*polycaprolactone* edo PCL) gelatinan oinarritutako matrizeen propietate mekanikoak hobetzeko gehien erabili izan diren polimero sintetikoak dira. Azido laktikoa optikoki aktiboak diren bi forma dituen molekula kirala da: L-laktida eta D-laktida. Molekula hauen polimerizazioz semikristalinoa den polimeroa sortzen da. Polimeroaren propietate mekanikoak pisu molekularra eta kristalinitate mailaren araberakoak izango dira. PLA degradazio tasa geldoa duen polimero biodegradagarria da, hainbat erabilpen medikoetarako ikertu izan delarik: farmakoen askapenerako sistemak, sutura xurgagarriak edota inplanteak [94]. Bestalde, PCL erresistentzia mekaniko altua eta hidrofilia baxua erakusten duen polimero sintetiko lineal hidrofobikoa da [92]. Bi polimero biodegradagarri hauek, propietate mekaniko egokiak erakusten dituzte eta erraz lortu eta prozesatu daitezke [95].

Azken urteetan, polimero natural eta sintetikoen nahasteak, hala nola gelatina eta PLA edo PCL nahasketak, asko erabili izan dira nanozuntz konposatuak prestatzeko [96]. Fabrikazio prozesu honetan, kontuan eduki behar da, gelatina/PCL skaffold hibridoekin egindako ikerketa lan batean ondorioztatu bezala, nanozuntz horiek morfologikoki uniformeak eta konposizio homogenoa izatea komeni dela [97]. Bestalde, nanozuntz hibridoak produzitzeko garaian material bakoitzak nahastean duen proportzioa garrantzitsua da. Izan ere, amaierako produktuaren mekanika eta gaitasun biologikoa horren araberakoa izango da. Ezaugarri mekaniko egokien eta zelula ama mesenkimalen (*mesenchymal stem cells* edo MSC) portaera optimoen arteko korrelazioa deskribatu izan da. Efektu honen arrazoiaren atzean, ezaugarri mekanikoak zelulen mikroingurune fisikoaren zati izatean egon daiteke, zelulen funtzioan eragin zuzena izanik [91].

Berriki, 100 eta 500 nm lodiera arteko zuntz lerrokatuz osatutako 3D matrizea diseinatu dute, berau sortzeko materialak gelatina eta azido poli (L-laktikoa) izanik. Elektroirute teknika erabiliz fabrikatu dira eta odol-hodien ingeniaritzan erabiltzera bideratuta daude. Entsegu ezberdinek frogatu dutenez HUVEC eta muskulu leuneko zelulen (*smooth muscle cells* edo SMCs) bideragarritasuna eta hazkuntza hobetu egiten da, gelatinaren edukia proportzionalki handitzerakoan. Emaitza horiek iradokitzen duten moduan, zelulen antolakuntza hobetu egin daiteke, skaffold-a osatzen duten zuntzen lerrokatzearekin. Hori dela eta, aipatutako formulazio hau erabilgarria izan daiteke funtsezko propietate mekaniko eta antolakuntza duten odol-hodietarako [89].

Hala ere, material konposatu hibridoen propietateen arteko sinergia eraginkorrak lortzeko, beharrezkoa da substratuen arteko interakzioak gertatzea. Horregatik, euren arteko lotura kimikoak sustatzea metodologia aproposa izan daiteke. Orain gutxi burututako ikerkuntza lan batean, fotopolimerizazio teknika erabiliz PCL eta gelatina kobalenteki lotzea lortu da, kartilago ehunera bideratutako inplanteen propietate mekanikoak hobetuz. Hori lortzeko, alde batetik PCL eta bestetik gelatina talde metakrilikoekin funtzionalizatu dituzte, eta ondoren euren arteko lotura bultzatu [54].

3.2 Sistema konposatu eroaleak

Eroaleak diren skaffold-ak biomedikuntzaren arlo ezberdinetan erabili izan dira: nerbio [98], bihotz [99] eta hezur [100] ehun ingeniaritzan, besteak beste. Ehun horietan, seinale elektrikoek berebiziko funtsio fisiologikoa burutzen dute, zelulen atxikipena eta desberdintzapena kontrolatuz. Hori dela eta, badirudi eroaleak diren materialen erabilera bertara zuzendutako skaffold-en garapenean itxaropentsua den estrategia dela [101]. Izan ere, material horiek bulkada elektrikoak bideratu ditzakete zeluletaraino, beraien funtzioetarako lagungarri izanik. Kinada elektriko hauek zelulen atxikipena, proliferazioa, migrazioa eta desberdintzapena hobetu ditzaketela ikusi da [102]. Egiaz, elektrikoki eroaleak diren skaffold-ak

zelula amen gordeleku modura aritzeko diseinatu izan dira, elektrikoki ehunaren birsorkuntza bultzatzeko. Zelula amen desberdintzapen espezifikoa gidatzen eta modulatzen duten eredu gisa jarduten dute, inongo indukzio faktoreren beharrik gabe [103].

Material eroaleak hiru kategoria nagusitan sailkatu izan dira tradizionalki: polimero eroaleak, karbono materialak eta metalak. Azken urteotan, material konposatu eroaleak diseinatu dira, eroale den material batek izan ditzaken mugak gainditzeko helburuarekin. Horrela, materialen erredimendu orokorra hobetzea lortzen da, efektu sinergikoak bilatuz. Material eroale hauetako asko jatorri naturaleko polimeroekin konbinatu izan dira, hala nola gelatinarekin, eroankortsun bikaina duten sistemak lortuaz [104].

3.2.1 Polianilina

Polianilina (*polyaniline* edo PANI), baldintza azidoetan gertatutako anilinaren polimerizazioz sortutako produktu oxidatiboa da, medikuntza birsortzailean erabiltzeko gehien ikertu den polimero eroaleetako bat. Polimerizazio hori kimikoa edo elektrokimikoa izan daiteke, bere sintesia erraza da eta zelula espezifikoekin bateragarria dela frogatu da [98]. Hala ere, biobateragarritasuna handitu eta zelulen atxikipena hobetzeko, gelatina moduko materialekin konbinatu izan da, eroaleak diren hidrogel biobateragarriak sortzeko asmoarekin [105-108].

Adibide gisa, gelatina/PANI materialez osatutako sistema konposatuan ereindako H9c2 bihotzeko mioblastoen proliferazioa eta morfologia, zelulak kultibatzeko aurretratamendua duten plastikoetan izan zutenaren berdina lortu zela aipatu daiteke [109]. Emaitza horien arabera, eroalea den zuntzezko skaffold horrek ehunen ingeniaritzan erabiltzeko gaitasuna izan dezake, hazkuntza zelularraren euskarri moduan erabiliz.

Ildo beretik, gelatina eta PANI elkartuaz burututako beste ikerketa lan batean, Schwann zelulen proliferazioa eta *in vitro* biodegradagarritasuna aztertu zuten. Garatutako formulazioak eroankortasun, propietate mekaniko eta biobategarritasun profil egokiak zituela antzeman zuten. Skaffold eroale porotsu berritzaile hau gelatina eta kitosanoz osatuta zegoen nagusiki, PANI eta grafeno nanopartikulak gehitu zituztelarik, eroankortasun ezaugarriez hornitzeko. Formulazio hau, bereziki nerbio periferikoen sendaketera bideratuta dago [110].

Badirudi, Yibo eta kideek etorkizun handia izan lezakeen skaffold bioaktibo berri baten diseinua osatu dutela. Sistema horrek, propietate elektrikoak hobetuz, metakrilatutako gelatina skaffold-en biobateragarritasun eta propietate fisikoak (ur hartze gaitasuna, konpresio modulua, zelulen atxikipena eta hedapen gaitasuna) mantentzea ahalbidetu du. Sistema konposatu hibrido eroale hori garatzeko estrategia, anilina monomeroen polimerizazioa metakrilatutako gelatinaren baitan eragitea izan da. Gainera, ikerketa lan interesgarri honetan, garatutako hidrogel konposatuari, aurrez definitutako geometria konplexua emanarazi diote, estereolitografia teknikaren bitartez imprimatuz. Prozesu hau, fotosentikorrak diren hidrogel ezberdinei aplikatu ahal zaio, interfaze bioelektriko berrien garapena ahalbidetuz [111].

3.2.2 Karbonozko nanosubstratuak

Karbonozko konposatuak ehun ingeniaritzan erabiltzeko ikertu izan diren material eroaleak dira. Karbonozko nanohodiak (*carbon nanotubes* edo CNT) eta grafeno oxidoa (*graphene oxide* edo GO) dira horietako bi [103].

Material horiek propietate fisiko, kimiko eta mekaniko bereizgarriak dituztenez, zelula amekin bideratutako tratamenduetarako eta ehunen ingeniaritzan aplikatzeko material erabilgarriak direla kotsideratu izan da [112]. Konposatu hauek ezaugarri biologikoak hobetuko dituzten materialekin konbinatzea, gelatina kasu, ehunen ingeniaritzan erabiltzeko formulazioak optimizatzeko aparteko estrategia da, bereziki bihotz eta nerbio ehunen ingeniaritzarako [113,114].

3.2.2.1 Grafeno oxidoa (*graphene oxide* edo GO)

Grafenoa karbono atomoak bi dimentsiotako geruza bakarrean dituen nanomaterial makromolekularra da. Material honen bereizgarri, sendotasun eta malgutasun handiak dira. Gainera propietate fisiko-kimiko, propietate mekaniko, elektriko eta optiko bikainak dituela ikusi da, biobateragarria izanik gainera [115]. GO grafenoaren deribatu kimiko bat da, zeina grafitoaren oxidazio eta esfoliazioaren bidez lortzen den [116]. Talde funtzional hidrofiliko asko dituenez ingurune urtsuetan ondo sakabanatzen da. Horretaz gain, kimikoki aldadaketak eragin daitezkeenez bere egituran, aplikazio biomedikoetarako erabilgarria den materiala da [117].

Azken urteetan, formulazio hibrido mota ezberdinak garatu izan dira gelatina eta GOren konbinazioz, propietate eroale eta birsortzaileak dituzten sistemak lortzeko helburuarekin [118-123]. Material konbinazio hau miokardiora bideratutako terapia-geniko ez biral moduko sistema gisa erabili izan da. Odol-hodien sorrera eta bihotz ehunaren orbaintze prozesua bultzatu ahal izateko, gelatina metakrilatua eta GO nanokonplexuen konbinazioan oinarritutako hidrogel injektagarria ikertu dute. Nanokonplexu hau, GOko nanosubstratuz funtzionalizatutako polietileniminazko sistema bat da, zeina endotelio baskularraren hazkuntza faktorea (*vascular endothelial growth factor* edo VEGF) kodetzen duen genearekin konplexatu den. Formulazio hau erabiliz, terapia genikoa modu kontrolatuan eta lokalizatuan aplikatzea lortu da. *In vivo* eginiko ikerketetan ondorioztatu zen moduan, formulazio injektagarri honek aurrez infartatutako eremuko kapilare dentsitatea areagotzea lortu zuen. Gainera, behaketa honekin batera ekokardiografia probaren emaitzak hobetzea lortu zen [121]. Animaliekin eginiko azterlan honetan lortutako eraginkortasun terapeutiko eta biobateragarritasun egokiak kontuan hartuta, sistema konposatu horien garapena bihotzeko akutuaren terapien garapenerako aurrerapen garrantzitsu bat izan daitekeela ondorioztatu daiteke. Gainera, gelatina eta GO konbinatzen dituzten formulazioekin egindako zenbait ikerketetan ikusi ahal izan den moduan, GOren presentziak alde batetik bihotz-zelulen portaera erregulatzeko

beharrezkoak diren propietate eroale elektrikoak eransten ditu eta bestalde propietate mekanikoak hobetu eta matrizean poro osagarriak sortzen laguntzen du. Poro hauek lagungarriak dira nutrienteen eta zelulen metabolismoaren ondorioz sortutako hondakinen difusioa matrizean zehar errazteko [120].

Badira, gelatinazko matrizean GO gehitzeak indar mekanikoa handitu egiten dutela ondorioztatu duten ikerketa lan gehiago ere. Adibidez, beste azterketa batean egiaztatu zen bezala, GO gelatina eta HAn oinarritutako formulazioan sartzeak, indar mekanikoan (konpresio indarraren gorakada) eragina zuela ikusi zuten baina baita desberdintzapen osteogenikoan ere. Izan ere, giza jatorriko gantz-ehunetatik eratorritako MSCen desberdintzapena, osteogenikoa izatea bermatu zen. Desberdintzapen maila hori, medioan gehitutako osagarriekin lortzen denaren adinakoa izatea lortu zen [123].

Horren harira, Shin *et al.*-ek gelatina metakrilatua eta erreduzitutako GOz osatutako hidrogel konposatua garatu zuten, miokardio ehunaren ordezkoa sortzeko helburuarekin. Kasu honetan, kardiomiozitoak sistema konposatuaren barnean erein ziren, zelulen bideragarritasuna, proliferazioa eta heltzea hobetuz. Gainera, zelula hauen uzkurkortasuna indartsuago egin zen, soilik gelatinaz osatutako matrizeetan barneratutako zelulekin alderatuz [122].

Emaitza horiekin guztiekin, GO gelatinan oinarritutako matrizeei gehitzeak eroankortasuna ez ezik, formulazioaren propietate mekanikoak eta MSCen desberdintzapen espezifikoa hobetzen dituela ondorioztatu daiteke.

3.2.2.2 Karbonozko nanohodiak (*carbon nanotubes* edo CNT)

CNT jatorrri naturaleko polimeroekin konbinatu ohi izan diren karbonozko beste nanosubstratu mota bat dira, elektrikoki eroaleak diren egitura indartsuagoak diren formulazioak lortzeko baliagarriak izanik. Badirudi, CNTekin erlazionatutako zitotoxikotasuna, berauek erabiltzeko moduaren araberakoa dela, zelulen kultibo medioan suspentsioan mantenduz gero toxikoak direlarik. Aldiz, matrize batean inmobilizatzen badira, toxikotasuna galtzen dutela ikusi da. Eragin desiragaitz hauek sahiesteko, CNTen gainazala funtzionalizatu daiteke [124]. CNTek zelulen atxikipena hobetu eta zelulen morfogenesia eta seinaleztapen bideak aldarazten dituzte. Gainera, materialen degradazio tasa geldoagoa izatea bideratzen dute, baita propietate mekanikoak modulatu ere [125].

Azken ikerketa lan batean ondorioztatu zen moduan, horma-anitzeko CNT barnean dituzten gelatinazko nanozuntz hibridoez osatutako skaffold-en garapena erabilgarria izan daiteke muskulu hodien eraketa bultzatzeko. Egitura hauek gihar-ehunen ingeniaritzan eskatzen diren funtzioak hobeto bete ditzaketela aurreikusi dute. Muskulu-eskeletiko ehuna simulatuko duen sistema funtzionala diseinatzerako orduan, oso garrantzitsua da inguru horretako ECM moduan, bertan ereindako mioblastoak lerrokatzea. Zelula hauen lerrokatzea hobetu eta euren uzkurkortasun gaitasuna handitzeko, lerrokatutako nanozuntzak erabiltzea

proposatu dute Ostrovidov eta lankideek. Lan horretan, horma-anitzeko CNTak lerrokatuta dituen gelatina skaffold hibridoa diseinatu dute [126]. Sistema berri horrekin, muskulu hodien formakuntza bideratzea posible izan zen, mekanotransdukzioarekin erlazionatutako geneen aktibazioa eraginez. Izan ere, bi materialen propietate sinergikoek C2C12 mioblastoen lerrokatzea lortu zuten, miozuntz funtzionalak sortu zituztelarik [127].

Antzerako estrategia bat jarraituz, osagai polimerikoak, gelatina eta glizerolaren eratorri bat, erresistenteak eta malguak diren CNTekin konbinatuz bihotz-ehunaren ingeniaritzan erabiltzeko sistema konposatua garatu dute. Elektroirute teknika erabili zuten nanozuntz hibridoak sortzeko eta bertan ereindako kardiomiozitoen uzkurkortasuna aztertu dute. Matrizean CNT gehitzeak, zelula horien uzkurkortasuna hobetzea eragiten du. Hori dela eta, propietate mekaniko eta elektriko egokiak aurkezten dituenez, bihotz ehunetan injertu gisa erabiltzeko baliagarria izan liteke [128].

3.3 Molekula bioaktiboen askapen kontrolatua

Zauritutako ehun baten orbaintzea edota ordezkapen eraginkorra lortzeko orduan, biomaterialek eta zelulek ez ezik, biologikoki aktiboak diren molekulek ere berebiziko garrantzia dute. Molekula hauek mikroingurune naturala mimetizatzen lagunduko dute, zelulen arteko komunikazioa bideratuz. Ezaugarri aproposak dituen materiala, zelula eta seinaleztapenerako biomolekulekin konbinatuz gero ehun ingeniaritzarako emaitza arrakastatsuak lortu ahal dira [129,130].

Seinaleztapen biomolekulak, oro har, hazkuntza faktoreak, zitokinak, kimio-erakartzaileak edo atxikipen proteinak izaten dira, era lokalean profil iraunkor bat jarraituaz euren forma aktiboetan askatzen direlarik. Hori dela eta, molekula horiek neurri handi batean zelulen portaeran eragiten dute. Hazkuntza faktoreak, zelulen gainazalean aurkitzen diten hartzaile espezifikoetara lotzen diren polipeptido gisa definitu daitezke, zelulen proliferazioa, ezberdintzapena, migrazioa, atxikipena eta geneen espresioa modulatzen dutelarik. Eragin zelular hauek, zelula-barneko kaskada konplexuen aktibazioaren ondorioz lortzen dira [9]. Horregatik, hazkuntza faktoreen erabilerak inplantatutako zelulen proliferazioa eta ezberdintzapena bizkortzen du, ehunaren birsorkuntza prozesua bultzatuz. Hala ere, baldintza fisiologikoetan beraien degradazio kimikoa edota entzimatikoa oso denbora laburrean gertatzen da. Arrazoi hori dela eta, faktore hauek erabiltzeko, euren aktibitate biologikoa luzatuko duten dosifikazio formak diseinatzea beharrezkoa da. Faktore hauen askapena denboran eta lekuan kontrolatzea lortuz gero, alde batetik proteina hauek birsortze prozesuan duten eraginkortasuna handitzea lortzen da, eta bestalde haiek sortutako eragin desiragaitzak sahiestea lortu daiteke [131].

Jatorri naturaleko polimeroetan oinarritutako hidrogelek, hala nola gelatina, ehun ingeniaritzarako bereziki egokiak izan daitezke, aldi berean funtzio bikoitza bete dezaketelako:

zelulen eramaile modura jarduteaz gain, uretan disolbagarriak diren konposatuen askapen iraunkorra baimendu dezakete. Gelatina polimero hidrofilikoa izanik, printzipio aktiboen askapenerako eramaileen gainazala estaltzeko erabili daiteke, opsonizazioa sahiestu eta euren ur-disolbagarritasuna handitzeko [132].

Aurretik deskribatu den moduan, gelatinak karga isoelektriko ezberdinak izan ditzake eta horregatik bereziki interesgarria izan daiteke, kontrako karga duten proteinekin elkarrekintzak sortu ditzakeelako. Gelatinan oinarritutako sistemetatik gertatutako molekula bioaktiboen askapena, gelatina matrizearen degradazio eta ur hartzeko propietateen araberakoa izango da. Aldakortasun honek aukera ematen du, baldintza egokituak erabiliz, askapen profila moldatzeko. Aipatutako askapen profil hau gelatinaren jatorria eta pisu molekularraren araberakoa da eta, elkargurutzaketa gradua edota sistema konposatuak osatuz optimizatu edo doitu daiteke [20]. Printzipio aktiboen askapenerako biomimetikoak diren gelatinadun sistema konposatuak prestatzeko, jatorri naturaleko hialuronatoa [133,134], kitosanoa [135] edota zeta [136] erabili izan dira. Polimero sintetikoei dagokionez, azido poli(laktiko-ko-glikolikoa (p*oly(lactic-co-glycolic) acid* edo PLGA) [137], oligoetilenglikol fumaratoa [138] eta polipropilen fumaratoa bezalako materialak konbinatu izan dira gelatinarekin, molekula bioaktiboen askapena doitzeko.

Azken urteetan, formulazio bakar batetik hainbat hazkuntza faktore aldi berean askatzeko sistema konposatuak diseinatu dira. Zentzu honetan, gelatinazko hainbat dosifikazio sistema garatu dira hainbat biomolekularen askapena lortzeko, hala nola insulinaren-antzeko hazkuntza faktorea-1 (*insulin-like growth factor* edo IGF-1) edo hezur-proteina morfogenikoa - 2 (*bone morphogenetic protein-2* edo BMP-2) [12,140].

4. GELATINAN OINARRITUTAKO SISTEMAK

Gelatina biomateriala biomedikuntza eta farmazia arloetan erabili izan da jadanik. Material oso moldakorra denez gero, txerto birikoen garapenerako erabili izan da, difteria eta errubeola gaixotasunen aurkako immunizazio prozesuetan, gelatinak egonkortzaile funtzioak izan ditu. Gainera kapsula gogor eta bigun geihenak gelatinaz osatuta daude eta plasma hedatzaileen formulazioen osagai bezala maiz erabiltzen da gelatina [18]. Horretaz gain, terapia aurreratuetako formulazioetan erabiltzeko ere proposatu izan da, non zelulek eta biologikoki aktiboak diren faktoreek protagonismoa irabazten duten.

Material honen abantaila nagusietako bat, helburu anitzetarako diseinatutako formulazio mota desberdinetan material gisa erabiltzeko gaitasuna da. Zentzu honetan, hazkuntza faktore eta zelulen askapenerako baliagarriak diren gelatinazko nano eta mikropartikulak arrakastaz garatu izan dira. Gelatinan oinarritutako 3D skaffold-ak, ehunen birsorkuntza gidatzeko baliagarriak diren molekula bioaktiboen eta zelulen eramaile gisa oso erabiliak izan

dira. Formulazio hauen aldaera bat, elektroirute teknika bidez sortzen diren nanozuntzez osatutako 3D formulazioak dira. Azkenik, *in situ* gelifikatzen diren hidrogelen garapenerako material aproposa dela ikusi da, formulazio hauek injekzio bidez administratzen direlarik.

4.1 Partikulak

Azken urteetan, gelatinazko partikulak ehun ingeniaritza esparruan ez ezik, aplikazio askotarako eramaile gisa ere erabili izan dira. Gelatina partikulak mota askotako printzipio aktiboekin kargatu izan dira, hala nola, minbiziaren aurkako farmakoak (metotrexatoa, zitarabina, doxorrubizina…), didanosina, klorokina, errifanpizina, isoniazida edo ibuprofenoa. Kasu hauetan, gelatinak funtzio ezberdina bete dezake formulazioaren baitan. Zenbait kasutan, farmakoaren askapena iraunkorra izatea baimendu dezake, beste kasuetan aldiz, printzipio aktiboaren eragin desiragaitz toxikoak gutxitu ditzake, edota profil farmakozinetiko eta aktibitate farmakologikoa hobetu. Gelatinazko nanopartikulak terapia-geniko ez birala burutzeko bektore gisa ere erabili izan dira [141].

Gainera, tamaina ezberdineko gelatina partikulak, bai mikrometrikoak baita nanometrikoak ere, medikuntza birsortzaileera bideratutako formulazioetan gehitu izan dira, ehunen ingeniaritzarako helburuak betetzeko elementu garrantzitsuak izanik.

4.1.1 Nanopartikulak

Nanoteknologia zientziaren hainbat esparrutan interes handia piztu duen eremua da, sektore biomediko eta farmazeutikoan hainbat aplikazio esploratu izan direlarik [142]. Hainbat emanbide erabiliz administratu daitezkeen nonosistemak diseinatu dira, hala nola nanopartikulak, nanozuntzak edo liposomak. Biologikoki egonkorrak eta bateragarriak dira, eta hainbat eta hainbat biomolekula kapsularatu daitezke euren barnean [143].

Nanopartikula polimerikoak jatorri natural edo sintetikoko materialez osatuta dauden 1 eta 1000 nm arteko tamaina izan ditzaketen sistema koloidalak dira. Partikula hauek izaera oliotsu edo urtsua duen barne fasea polimeroz izaten dute estalita. Talde honen barne, ezberdinak diren hainbat sistema aurkitu daitezke, nanoesferak eta nanokapsulak besteak beste. Nanoesferak, bertan kargaturiko farmakoa uniformeki dispertsatuta aurkitzen den sistema matrizialak dira. Aldiz, nanokapsulek besikula bat osatzen dute [144]. Nanopartikulek, oro har, hazkuntza faktoreen eramaile gisa jarduteko balio dute; beraien barnean konposatu bioaktiboak kargatu eta ehunen ingeniaritzan erabilitako formulazioen eraginkortasuna handitu dezakete. Kapsularatutako molekulen funtzio biologikoak hobetu daitezke, eskala nanometrikoan antolatutako askapen iraunkorreko sistemak diseinatuz. Tamaina nanometrikoa duten partikulek molekula aktiboa babestu eta euren bioerabilgarritasuna handiagoa izatea baimentzen dute, gainazal-bolumen ratio handiari esker [145]. Oro har, nanopartikulak diseinatu eta prestatzea erraza dela esan daiteke. Literaturan, gelatinazko nanopartikulak prestatzeko hainbat eta hainbat metodo daude deskribatuak jadanik. Bi-urratsetako desolbatazioa,

koazerbazio bakuna, disolbatzailearen lurruntzea, mikroemultsioen prestaketa, nanoprezipitazioa eta auto-mihiztadura (*self-assembly*) dira horietako batzuk. Teknika horien guztien deskribapen zehatza jadanik argitaratutako berrikuspen batean biltzen dira, bakoitzaren abantaila eta desabantailen eztabaida sakona osatuz [146].

Gelatinazko nanopartikulen modifikazio edo eraldaketak diseinatuz, sistema aurreratu ugari sortu daitezke. Orain gutxi burututako ikerkuntza lan batean, gelatina sukzinilo taldeekin eraldatu zen, giro tenperaturan disolbagarria den polimeroan bilakatuz. Gelatina eraldatu hori, heparinaren oxidazio prozesuan zehar sortutako talde aldehidoekin elkargurutzatu zen, toxikoak izan daitezkeen erretikulazio agente eragileen erabilpena sahiestuz, hala nola glutaraldehidoa. Gainera, nanopartikula hauek kaltzio ioien nukleaziorako euskarri moduan jardun ahal dute, HA kristalak sortuz. Horrela, nanopartikula mineralizatuak sortu daitezke, hezur-ehunaren ingeniaritzarako baliagarriak izanik [147].

Material sintetikoek aplikazio biomedikoetan erabiltzeko aurkezten duten desabantailarik nagusiena, euren biodegradagarritasun txikia da. Gelatina material horiekin konbinatuz gero, eragozpen hori murriztu egiten dela ikusi da. PCLaz eratutako nanozuntzezko skaffold-etan gelatinazko nanopartikulak gehitzeak, skaffold sintetiko horien degradazio denbora murriztu zuen. Izan ere, nanopartikula horien degradazioak, egituraren poroen tamaina handitzea eragiten du, zelulak egituran barneratzea erreztuz [148]. Horregatik guztiagatik, gelatinazko nanopartikulak dituzten formulazio aurreratuak garatu izan dira azken urteetan, ehun espezifikoen birsorkuntzara bideratu izan direlarik: hezurra [148], bihotz-ehuna [149] edota zaurien orbainketa [150].

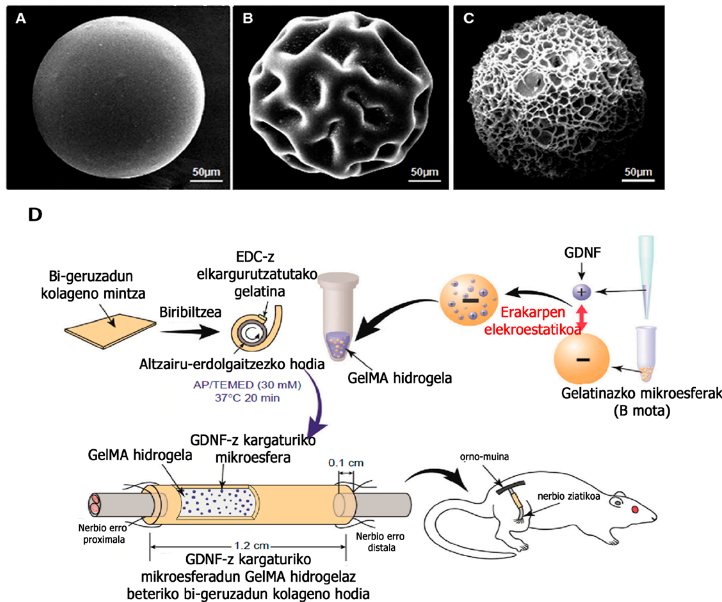
Orain gutxi burututako ikerlan batean, miokardioko infartu akutuko arratoi ereduetan, ehun kardiakoaren berezko erreparazioa bultzatu nahi zen. Horretarako, kardiomiozitoen biziraupena eta proliferazioa bultzatzen duten bi hazkuntza faktorez kargaturiko hidrogel hibridoa diseinatu zen. 180-255 nm-ko tamainadun nanopartikuletan 6-bromoindirubin-3-oxima eta IGF-1 faktorea sartu ziren, askapen iraunkorra lortu eta arratoien bihotz funtzioa hobetu zela antzeman zuten, kardiomiozitoen proliferazioa eta infartatutako eremuaren baskularizazioa areagotuz [149].

Faktoreen askapen profilaren gaineko kontrola lortzeak, hain zuzen ere, birsorkuntza eraginkorrerako beharrezko funtsezko elementua dirudi. Zentzu honetan, hainbat hazkuntza faktore angiogeniko ikertu dira zaurien orbaintzea sustatzeko. Alabaina, zauriak sendatu eta azalaren birsortze prozesu fisiologikoa imitatzeko, elkarrekin erabilitako biomolekula horien askapenak, zinetika fisiologikoarekin bat etorri behar dute. Horietako batzuk, fibroblastoen hazkuntza faktore basikoak kasu, zelula endotelialen erreklutazioa estimulatzen dute eta beste faktore batzuen zeregina odol hodi berrien engorkotzean datza. Profil zinetiko ezberdin horiek lortzeko, sistema bakar batean hainbat faktore barneratzeko zenbait metodo erabili izan dira.

Elektroirute bidez prestatutako nanozuntzetan faktoreen integrazioa zuzenean egiten bada, hauen askapena azkarra izango da hasieran; aldiz, gelatina nanopartikulen barnean kokatzen badira molekula aktiboak, euren askapena motelagoa eta iraunkorragoa izatea lortuko da. Horixe lortu zen, zaurien orbaintzea bultzatzera bideratutako hazkuntza faktore angiogeniko ugari zituen elektroirutearen bitartez sortutako nanozuntz konposatuetan [150].

4.1.2 Mikropartikulak

Gelatinazko mikropartikulak fabrikatzeko metodo erabiliena, orain arte, ur-olio emultsio prestaketa izan da, ondoren elkargurutzaketa prozesua burutuz. Hala ere, partikula horien fabrikazio prozesua optimizatzeko, ahalegin handiak egiten ari dira, gainazal ezberdineko formulazio aurreratuagoak lortuz (3A, 3B, 3C Irudiak) [151]. Zentzu horretan, gelatinazko mikroesfera konposatuak garatzeko metodo berritzaile bat porposatu da. Gainazal malkartsudun eta nanozuntzezko egitura duten 124 eta 136 µm bitarteko mikroesfera esferiko porotsuak termikoki eragindako emultsioen fase bereizketa bidez sortu dituzte [152].



3. Irudia. Gelatinazko mikropartikulak ehun ingeniaritzarako. (A-C) Azalera ezberdineko gelatinazko mikropartikulen SEM irudiak. (A) gelatinazko mikropartikula leuna; (B) zulodun mikropartikula; (C) barrunbe anitzeko mikropartikula. (D) Arratoietan nerbio ziatikoaren hazkuntza bultzatzeko, glia-ko zeluletatik eratorritako faktore neurotrofikodun (*glial cell-derived neurotrophic factor* edo GDNF) mikroesferaz kargaturiko gelatina-metakrilamida hidrogelez estalitako kolageno bigeruza egituraren irudi eskematikoa. A-C irudiak [151] erreferentziatik eta D irudia [160] erreferentziatik baimenerakin egokituak daude.

Gelatinazko mikropartikulen garapenarekin, ehun ingeniaritza alorrean molekula bioaktiboak askatzeaz gain, terapia zelularra burutzeko ahaleginak ere egin izan dira. Mikropartikula hauen laguntzaz zelulen askapena bideratzeko, hainbat estrategia ezberdin jarraitu izan dira, hala nola, partikulen gaineko zelulen atxikipena gauzatzea. Metodologia honekin , ehun espezifiko batean ezarritako zelulen kopurua nabarmen handitzea lortu daiteke. Horrela, gelatinazko mikropartikulen kanpo gainazalean atxikitako zelulen administrazioa burutuz, miokardio iskemikoan bildutako zelula kopurua, inongo euskarririk gabe zelulak injekzio soilez administrazean baino 10 aldiz handiagoa izan daitekeela ikusi da. Gainera, mikropartikuladun formulazio hau ehun kardiakoan kateterren bidez administratu daiteke, bihotzeko kirurgia inbaditzaileak sahiestuz [153].

Bestalde, hainbat eta hainbat dira gelatinazko mikropartikuletan kapsularatuak izan diren zelula motak. W. Leung eta lankideek ainguraketa-menpekoak ez diren (*non-anchorage dependent*) zelulak kapsularatzeko egokia den hidrogel aurrerakoia diseinatu dute. Zelula mota hauek, zelulen isloteak garatzeko joera erakusten dute. Sistema honetan, kondrozitoz kargaturiko gelatinazko mikropartikulak alginatozko hidrogel batekin konbinatu dira. Mikroesferak ur-olio emultsio prozesuaren bidez eratzen dira, baina elkargurutzaketa bultzatzeko inongo tratamendu kimikorik gabe. Hori dela eta, tenperatura 37 °C-tara igotzean, mikropartikulak disolbatu egingo dira eta barnean daramatzaten zelulen askapena gertatuko da, prozesuan sortutako poroetan kokatuz. Kasu honetan, gelatinazko mikropartikulek bi funtzio ezberdin betetzen dituztela esan daiteke: alde batetik, zelulen eramaile banangarriak dira, eta bestalde poroen sorkuntza sustatzen dute, gelatinazko hidrogelaren baitan nutriente eta metabolismo zelularraren hondakinen difusioa baimenduko duten barrunbeak sortuz [154].

Terapia zelularren eraginkortasuna handitzeko proposatutako beste estrategia bat, gelatinazko mikropartikula hutsak zelulak dituen hidrogelean murgiltzea izan daiteke. Mikropartikula hauek, entzimatikoki degradagarriak izanik, poroak sortzeko bidea irekiko dute, zelulei atxikipenerako guneak eskainiz jatorri sintetikoko hidrogeletan. Izan ere, MSC zelulen epe luzerako bideragarritasuna hobetu egiten dela ikusi da, formulazioan 50-100 µm arteko gelatinazko partikulak gehituz gero, zelula horiek ainguraketa-menpekoak (*anchorage-dependent*) baitira. Halaber, gelatinazko mikropartikulek zelulengan eragin espezifikoagoa izan dezaketela ere deskribatu da, adibidez ezberdintzapen osteogenikoa modulatu eta hidrogelen *in vitro* mineralizazioa sustatu [155,156].

Azken urteetan, gelatinazko mikropartikulak zelula-amen agregatuetan erabili izan dira, hazkuntza faktoreen askapena kontrolatuz desberdintzapen prozesua bideratzea lortuz. Gelatinazko mikropartikulak zelula-esferoideetan txertatzeak ez du funtsean zelulen antolamendua aldatzen, baina zelulen mikroingurua zurrunagoa izatea eragiten duenez, zelula-amen ezberdintzapena gogortasun handiko ehunetan aurkitzen diren zeluletara

bideratzea lortzen da, hala nola hezurra [157]. Enbrioi zelula-amen agregatuetan aipatutako mikropartikulak barneratzean, morfogenesi mesenkimala eta ezberdintzapena bultzatu daitezke. Izan ere, mikroesferek MMPen espresioa areagotzea eragiten dute, zeintzuak ECMren birmoldaketa egiten dutenez, ezberdintzapen zelularra doitzeko aukera ematen duen [158].

Gelatinazko mikropartikulak erabiliz zelula-ama pluripotenteen agregatuetan ezberdintzapena bideratzeko beste estrategia bat, hazkuntza faktore espezifikoak barnean dituzten partikulak erabiltzea izan daiteke. Kasu hauetan, morfogenoaren gradientea sortuko da agregatuaren baitan. Gelatinazko mikropartikulak hezur-proteina morfogenikoa - 4 (*bone morphogenetic protein-4* edo BMP-4) hazkuntza faktorearekin kargatuta administratuz, pluripotenteak ziren enbrioi zelula-amen esferoideetan, espazioan kontrolatutako ezberdintzaketa lortu zen. Faktore honen karga 125 ng-koa izan zen, mg mikropartikula bakoitzeko. Seinaleztapenerako faktore hauek, 3D agregatu zelularretan barrena difusioz garraiatzeko zailtasunak izaten dituzte, ezberdintzapen prozesua orotara homogeneoa izatea oztopatuz [159].

Gainera, gelatinazko mikroesferek hazkuntza faktoreak eramateko duten gaitasuna dela eta, oso erabiliak izaten ari dira ehunen ingeniaritza aplikazioetan, hala nola angiogenesi terapeutikoa bultzatu, kartilago, hezur edota nerbio-ehunen ingeniaritza bideratu eta baita miokardioko infartu akutuen terapietan ere [36]. Adibide gisa, gliako zeluletatik eratorritako faktore neurotrofikoz kargatutako gelatinazko mikropartikulak nerbio ziatikoaren hazkundea sustatzeko garatu dira [160]. Formulazio hau gelatinan oinarritutako sistema konplexu moduan deskribatu daiteke, izan ere material hau hainbat modutan formulatuta aurkitzen da sistema bakar batean. Kasu honetan, gelatina aipatutako mikropartikulak osatzeko ez ezik, hauek sakabanatuta dauden hidrogela osatzeko ere erabili da (3. Irudia D).

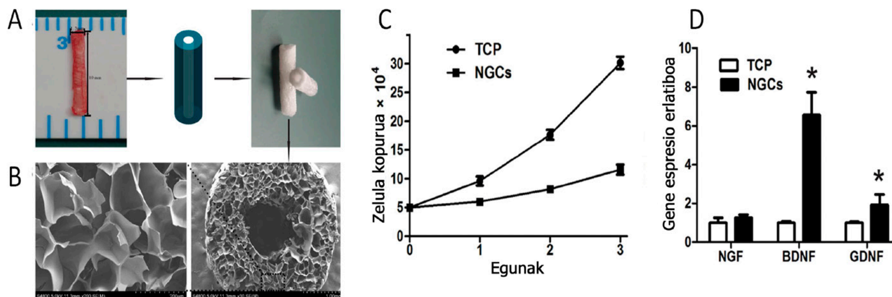
Bestalde, hainbat hazkuntza faktore formulazio bakar batean konbinatuz gero, euren eraginkortasun terapeutikoa handitu egiten dela ikusi da [161]. Hori dela eta, IGF-1 eta BMP-2 hazkuntza faktoreak elkarrekin erabiliz gero, fosfatasa alkalinoaren (*alcaline phosphatase* edo ALP) aktibitatea handitu egiten dela ikusi da. Entzima honen aktibitatea ezberdintzapen osteoblastiko goiztiarraren markatzaile dela kontsideratzen da. Bi hazkuntza faktore hauen askapen sekuentziala, IGF-1-z kargaturiko gelatinazko mikropartikulak BMP-2 daukan kitosanozko gel batean kapsularatuta lortu daiteke, adibidez. Molekula hauen askapena, mikropartikulen elkargurutzaketa mailaren, hidrogelaren baitan partikulen sakabanaketa eta proteinen eta material eramaileen arteko elkarrekintzen araberakoa izango da [162].

Horrez gain, gelatinazko mikropartikuletatik printzipio terapeutikoen askapena, alde batetik difusio prozesuaren ondorioz gertatuko da, baina baita partikula beraien biodegradazio entzimatikoari esker ere. Zentzu horretan, iskemia jasan duten arratoien garunetan, gelatinasa entzimen espesioa induzitu egiten dela deskribatu dute. Hori dela eta, kasu horietan

mikropartikula hauen degradazioa azkarrago gertatzen da. Egoera hau medio, osteopontinaren efektu neurobabesgarria handiagotu egiten dela ikusi da gelatinazko mikropartikulak erabiliz administratuz gero, askapena azkarragoa eta iraunkorragoa lortzen delarik [163].

4.2 Skaffold-ak

Skaffold-ak aurrez eratutako 3D egitura porotsu inplantagarri gisa definitzen dira, birsorkuntza prozesuaren euskarri funtzioa izateko diseinatzen direlarik. Gaur egun, aurrez aipatutako eskakizun guztiak betetzen dituzten skaffold-en diseinua eta ekoizpena erronka bat izaten dirau. Hala ere, gelatina eskakizun horiek guztiz betetzeko hautagaia izan liteke, bere molekulan aldaketak burutuaz edota aurreko ataletan deskribatu moduan material ezberdinekin konbinatuz matrize hibridoak sortuz. 3D egitura horietan zelulak eransteko hainbat metodo jarraitu daitezke. Esaterako, aurrez prestaturiko egituren gainazalean zelulak erein eta kultibatu daitezke. Berriki amaitutako ikerketa lan batean, gantz ehunetik eratorritako zelula-amak gelatina metakrilatuz osatutako nerbio-gidaritzarako kanaletan kultibatu ziren. Zeluladun skaffold hauek arratoien nerbio ziatikoaren ezaugarrietan oinarrituz diseinatu eta zeharkako 3D inprimazio teknikaren bitartez fabrikatu dira (4. Irudia) [164]. Halere, ez da erraza zelulak egitura osoan zehar uniformeki banatuta dituen skaffold-ak lortzea, zelula gehienak egituraren gainazalean geratzen baitira. Beste aukera bat, zelulak egituren fabrikazio prozesuan zehar barneratzea izan daiteke. Horretarako, zelulen bideragarritasuna mantendu ahal izateko, konposatu toxikoak eta zelulentzat bortitzak izan daitezkeen baldintzak sahiesten dituzten protokoloak erabili behar dira [165].



4. Irudia. Nerbio periferikoen birsorkuntzarako zeluladun gelatinazko nerbio-gidaritzarako kanalak *(nerve-guidance conduits* edo NGC). (A) Nerbio ziatikoaren diametro neurketa, kanalen diseinua eta fabrikazioa burutzeko. (B) Nerbio-gidaritzarako kanalen SEM irudiak. (C) Gantz ehunetik eratorritako ama zelulen (ASCs) proliferazio analisia, zelulen hazkuntzarako poliestireno (t*issue culture polystyrene* edo TCP) edo NGC sisteman zelulak kultibatu eta 1, 2 eta 3 egunen ondoren. (D) Erein ondorengo bigarren egunean ASCs zeluletan faktore neurotrofiko nagusien geneen espresioa: nerbioen hazkuntza faktorea (*nerve growth factor* edo NGF), garunetik eratorritako faktore neurotrofikoa (*brain-derived neurotrophic factor* edo BDNF), gliako zeluletatik eratorritako faktore neurotrofikoa (*glial cell-derived neurotrophic factor* edo GDNF). *p<0.05 TCP taldearekin alderatuz. [164] erreferentziatik moldatua.

Farmakoen askapenerako diseinatutako skaffold-en kasuan, egitura osoan zeharreko biologikoki aktiboak diren konposatuen sakabanaketa homogeneoa lortzea beharrezkoa da, hazkuntza faktoreen askapen iraunkorra lortu eta hasierako bapateko askapen efektua (*burst effect*) sahiesteko [166].

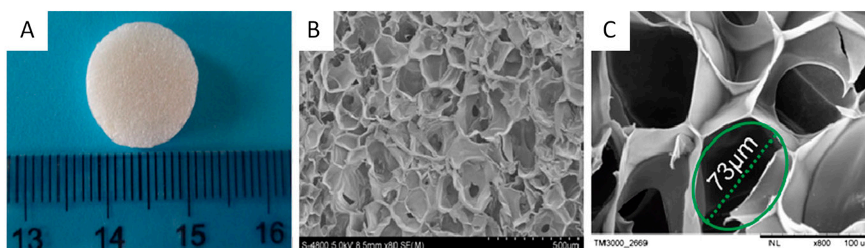
Oro har, 3D matrizeak sortzeko teknikei dagokienez, hainbat metodologia aplikatu izan dira. Disolbatzaile isurketa/partikulen lixibiatzea, liofilizazioa, fase banaketa, zuntzen lotura, fusiozko prozesaketa, elektroirutea eta ereduketa azkarra dira skaffold-ak fabrikatzeko erabilitako prozesamendu nagusiak [167]. Gelatinazko skaffold-en fabrikazioari dagokienez ere, teknika hauek erabili izan dira. Hala ere, hainbat ikertzaile teknologia horien aldaketak [168] edota konbinaketak [169,170] egiten saiatu dira. Zentzu horretan, propietate mekanikoen gradientea duten skaffold-ak sortu dira, HA kontzentrazio ezberdinetan duten gelatina eta kolageno nahasketak bata bestearen gainean pilatuz [171]. Amadori S. eta lankideek antzeko estrategia bat erabiliz ordezkapen osteokondrala burutzeko geruza ezberdinez osaturiko egitura hibridoak diseinatu dituzte gainjartze metodoaz baliatuz [172].

Ehunen ingeniaritzaren esparru ezberdinetara zuzendutako gelatinan oinarritutako skaffold-ak ekoizteko ahalegin handiak egin diren arren (zaurien orbaintzea [173], kartilagoa [174,175] eta larruazala [176] artean), badirudi hezurren birsorkuntza alorrean egin direla aurrerapen esanguratsuenak (5. Irudia) [177]. Gelatinan eta hainbat konpositeetan oinarritutako 3D egiturak arrakastaz aplikatu izan dira hezur-ehunen ingeniaritzan.

Ahalegin handi horien erakusgarri dira, gelatinazko matrizeetan osteoblastoetara ezberdintzatzeko gaitasuna erakutsi duten zelula mota ezberdinekin buruturiko *in vitro* zein *in vivo* ikerketak. Helburu horretarako gelatina egituretan ereindako zelula motei dagokionez, MC3T3-E1 sagu osteoblastoak, giza osteoblasto primarioak, untxien gantz jatorriko MSC eta giza hortz-pulpatik eratorritako zelula-amak [178-182] dira horietako batzuk. *In vitro* egiten diren entseguen artean, zelulen atxikipena eta proliferazio ikasketa, ALP aktibitatearen zenbaketa, hezurraren espezifikoak diren geneen espresioa edota tindatze immuno-histokimikoak izaten dira.

Euskarri horien hezur-birsorkunztarako potentziala *in vitro* frogatu ondoren, animalia eredu ezberdinekin hainbat ikerketa egin dira *in vivo*, klinikara transferitzeko urratsa egin aurretik, beharrezkoak diren frogak osatzeko. Hezur birsortzea ebaluatzeko animalia-eredu erabilienetariko bat, neurri kritikoko arratoien garezur-gangako defektua da [76,183-185]. Hala ere, osifikazioa sustatzeko gelatinan oinarritutako matrizeen gaitasuna, beste hainbat animalia eredu erabiliz ere probatu izan da; hala nola, untxien tamaina kritikoko ulna eredua [186] edo X-izpiekin irradiatutako animalia ereduak. Azken animalia eredu honen bidez, minbizia tratatzeko erabilitako X-izpien erradiazioaren ondorioz gertatutako hezur-sendaketa gaitasunaren hondatzea simulatu nahi da. BMP-2 eta hezur-muin autologoz

kargaturiko gelatinazko skaffold-ak egituraren birsorkuntza bideratu zuen, irradiatutako hezur akats segmentatuan [187].



5. Irudia. Kartilago ehuna mimetizatzeko erabilitako kitosano/gelatinaz osatutako skaffold-a. (A) Morfologia osotasunean; (B) Skaffold hibridoen gainazalaren topografia eta poroen estruktura; (C) Hidrogel konposatuaren gainazaleko 73 µm tamainako poroa. [175] erreferentziatik, baimenarekin moldatua.

4.2.1 Gelatinan oinarritutako elektroirundako skaffold-ak

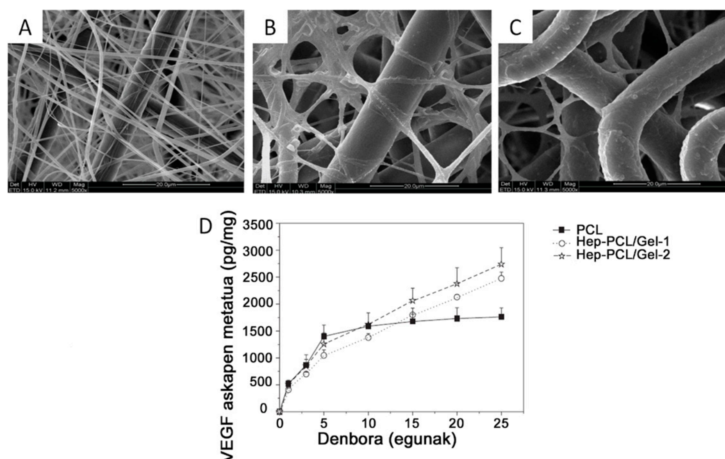
Elektroirute teknikaren bidez sortutako nanozuntzek, oso erabilera anitzak izan ditzakete, hainbat arlotan aplikazio sorta zabala burutuaz, esate baterako, eremu biomedikoa. Azken urteetan, tamaina nanometrikoak dituzten materialez osatutako arkitektura biomimetikoen ekoizpenak interes handia piztu dut. Elkar-lotutako zuntzekin osatutako 3D skaffold-ak sortu daitezke, ECMren egitura naturala imitatzeko. Egitura hauek oso porotsuak izaten dira, elkar konektatutako poroak izaten dituztelarik. Elektroirutea jatorri natural edo sintetikoko polimeroetatik abiatuz nano edo mikra tamainako zuntz meheak sortzeko balio duen teknika da [188]. Metodologia hau 1930 urteaz geroztik ezagutzen da eta ordutik, fabrikazio proezuan aurrerapen ugari lortu dira [189]. Teknologia honekin, 50 nm eta 10 µm arteko zuntzak fabrikatu ahal dira [190]. Fabrikazio prozesu hau polimero ezberdin askorekin buru ahal da, baita hainbat polimeroren nahasteekin ere.

Prozesua burutzeko zehaztu behar diren aldagaiei dagokienez, hiru taldetan sailkatu daitezkeen hainbat faktore daude: polimero disoluzioaren berezko propietateak, prozesuarekin erlazionatutako parametroak eta inguruko baldintzak. Aldagai guzti hauek, amaierako zuntzen kalitatea eta ezaugarriak baldintzatuko dituzte, baita zuntzek osatuko duten sarearena ere [191]. Morfologikoki uniformeak eta konposizio homogeneodun nanozuntzak ekoiztea oso garrantzitsua da, zelulen atxikipena eta proliferazioan eragina izango baitu [192].

Gelatinaren kasuan, gelatina molekulek disoluzioan aurkeztutako konformazioa oso faktore garrantzitsua izango da. Elektroirutearen bitartez gelatinazko nanozuntzak sortu ahal izateko, gelatina zorizko espiral edo "*random coil*" konformazioan egon beharko du disoluzioan. Disolbatzaile izaeraren arabera gelatinazko nanozuntzak prestatzeko bi modu daude.

Gelatina disolbatzeko ura erabiltzen denean, disoluzioaren tenperatura likido-gel trantsizio puntutik gora berotu beharko da. Izan ere, giro tenperaturan disoluzio horrek biskositate handiegia izango du beraren irutea gauzatzeko. Gainera, disoluzio urtsuaren gainazal tentsio handia dela eta polimero zurrusta ezegonkortu eta tanten formakuntza oztopatu dezake, fabrikazio prozesua zailduz. Gelatina gordailua sortzeko, disolbatzaile organikoak erabiltzea aukera eraginkorra da, gelatina egituraren helize hirukoitza ezegonkortu eta beronen elektroirutea baimenduz. Azidoak diren disolbatzaileak (azido azetiko [193] edo formikoa), trifluoroetanola, dimetilsulfoxidoa, etilenglikola edo formamida gelatina disoluzioen elektroirutea hobetzeko erabili izan diren disolbatzaileak dira [189]. Gelatinazko zuntzez osatutako skaffold-ak zaurien orbaintze prozesuan [194], nerbio [195], kartilago [196,197], hezur [198,199], azal [200], ehun kardiobaskular [201] eta begien [202] ehun ingeniaritzan aplikatu izan dira.

Azken urteetan, nanozuntzezko skaffold aurreratuak fabrikatu izan dira geruza anitzeko sareak [203], nukleo/estalki egiturak [204,205], biologikoki aktiboak diren konposatudun nanozuntzak edota ezaugarri mekaniko hobetuak dituzten egiturak [191] diseinatuz. PCL/gelatina zuntzezko skaffold hibridoekin egindako azterlan batean, VEGFaren askapen iraunkorra posible izan zen gelatinazko zuntzak heparinarekin funtzionalizatuz [206] (6. Irudia). Porositate handiagoa duten skaffold-en garapena elektroirute teknika eta gas aparra/gatzen lixibiazio prozesuak konbinatuz lortzea posible da. Skaffold hauek krater itxurako egiturak dira, sarean zeharreko giza MSC zelulen proliferazio eta infiltrazioa areagotzea lortzen delarik [207].



6. Irudia. PCL/gelatina sistema konposatuaren elektroirundako nanozuntzez osatutako skaffold-a. (A) PCL/gelatina nanozuntzen SEM irudia; (B) Heparinizatutako PCL/gelatina nanozuntzen SEM irudia; (C) Fosfatozko tanpoian murgilduta 14 egunez heparinizatutako PCL/gelatina formulazioaren degradazio erakusgarri den SEM irudia; (D) Heparinizatutako PCL/gelatinaz osatutako skaffold-etatik gertatutako VEGF faktorearen *in vitro* askapena, PCL-z soilik osatutako skaffold-ekin alderatuz (n=3). [206] erreferentziatik moldatua.

4.3 *In situ* gelifikatzen diren formulazioak

In situ gelifikatzen diren formulazioak, ehunen ingeniaritza esparruan emaitza onak lortu dituzten injekzio bidezko mentu moduan definitu daitezke. Sistema hauek, aurrez eratutako skaffold-ekin alderatuz hainbat abantaila eskaintzen dituzte. Alde batetik, minimoki inbaditzailea den emanbidez administratu daitezke helburu den ehunean, ebakuntza kirurgiko konplikatuak sahiestuz, gaixoaren onespena hobetuz [208]. Gainera, sistema horien jariakortasuna dela eta, aldi batez konplexuak eta forma irregularrak diuzten akatsak ordezkatzeko gaitasuna izan dezaketela erakutsi dute [209].

In situ eratzen diren hidrogel injektagarriak, baldintza fisiologikoetan gelifikazioa lortzeko gai diren hainbat metodologia erabiliz formulatu daitezke. Edonola ere, diseinu eraginkorrak lortzeko erreakzio kimiko horiek espezifikoak eta azkarrak izan behar dute. Hidrogel injektagarriak elkagurutzaketa kimiko bidez, elkarrekintza elestrostatiko bidez, auto-mihiztadura (*self-assembly*) estatregiak aplikatuz edota estimulu-erantzun metodoez baliatuz prestatu daitezke [210].

Mekanikoki sendoak diren gelatinan oinarritutako hidrogel injektagarriak, "*Host Guest Macromer*" elkargurutzaketa teknika erabiliz prestatzea proposatu izan da berriki. Makromero hau, hidrogelaren prestaketarako aitzindaria da, gelatinaren hondar aromatikoen eta fotopolimerizagarria den β-ziklodextrina akrilatuen artean sortutako konplexua, hain zuzen ere. Polimerizazioa UV irradiazioarekin hasten da eta gelatina kateak fisikoki elkargurutzatzen dira. Estrategia honekin ezaugarri bio-itsaskorrak dituen hidrogela sortu daiteke, farmako hidrofobikoen askapena ez ezik, zelula amen ezberdintzapenerako euskarri izanik ere [211].

Sandeep T. *et a*l.-ek gelatina molekuletan aldaketak burutuz, injektagarria den klik-elkargurutzaketa bidezko hidrogela garatu dute. Hidrogel hauek minutu gutxiren barruan osatzen dira, kanpoko energia, katalizatzaile edo erreakzio abiarazlerik gehitu gabe. Gelatina molekulan tetrazina eta norborneno talde funtzionalak gehituz, alderantzizko Diels-Alder klik erreakzioa eragiten da. Bioortogonalki elkargurutzatutako gelatinan oinarritutako hidrogelak sortzen dira, erreakzio horren emaitza moduan [212]. Orain gutxi, metodologia hontaz baliatuz, *in situ* gelifikatzen diren gelatinan oinarritutako mentuak garatu izan dituzte [213].

Gisa honetako hidrogelak, xede den ehunean zelulak ezartzera [214], edota hazkuntza faktoreen askapen iraunkorra lortzera bideratuta egon daitezke; hala nola, BMP-2-aren dosifikazioa arratoien garezurreko birsorkuntza prozesuan [215]. Gainera, injektagarriak diren hidrogelak erabiliz zelulen erantzun funtzionalak 3D ingurunean ikertu daitezke. Horrela, *in vitro* eginiko ikerketak errealitatean ehunetan topatzen duten ingurunera egokitzea lortu daiteke [216].

5. ONDORIOAK

Ehunen erreparazioa erregulatzen duen orbaintzearen mikroingurunea guztiz ulertzeak, maila akademikoan eginiko aurrerapenak klinikara iristeko beharrezko urratsak ematen lagunduko luke. Neurri handi batean, translazio horren arrakasta-eza, askotariko molekula biologikoek osatutako seinaleztapen prozesu konplexuak islatzeko zailtasunagatik izaten da.

Berrikuspen honek, gelatinak biomaterial gisa ehunen konponketan eta birsorkuntzan erabiltzeko aurkezten duen potentzialaren ikuspegi orokorra eskaintzen du. Aurrerapen esanguratsuak egin diren arren, lan asko dago aurretik biomaterial hau errutinazko praktika klinikoan txertatzeko. Orain arte, gelatinaren egituran eraldaketak egitea eta hainbat material konbinatuz sistema konposatuak sortzea, ehunen ingeniaritzarako eramaileak formulatzeko estrategia egokiak direla frogatu da. Hala ere, sistema hauen bioaktibitatea zelulen epe luzeko bideragarritasuna baimenduz hezur, bihotz edota nerbio-ehunen ordezko gisa jarduteko, ikerketa gehiago behar dira.

Gelatina ehun ingeniaritzako biomaterial gisa jarduteko eginiko etorkizuneko ikerketak, gelatinaren elkargurutzaketa prozesua hobetuz, biomaterialaren berezko propietate egokiak eta biobateragarriak mantentzera bideratu beharko litzateke. Klinikarako translazioa lortzeko erronka nagusietako bat, garatutako sistemen tamainen egokitzean datza. Hori dela eta, gelatinarekin eginiko ikerlanen norabidea, funtzionalak eta tamainaz egokiak diren ordezkoen fabrikaziorako teknologien garapenean egon daiteke.

Zorionez, baikorrak izateko arrazoiak badaude. Litekeena da formulazio eta fabrikaziorako metodologia berriek, gelatinan oinarrituriko sistemen aplikazio aukerak zabaltzea. Teknologia automatizatuak diseinatzea eta fabrikazio gehigarri edo 3D bioinprimatze teknologia berrien erabilerarekin batera, oro har gelatinazko formulazioen azken ezaugarriak doitzea erraztu dezakete. Segurtasunarekin eta erreproduzigarritasunarekin erlazionatutako arauetan emandako aurrerapausoak, beharrezkoak izango dira gelatinan oinarritutako medikuntza birsortzailea etorkizunean klinikan aplikatzeko. Aurrerapen horien eta beste batzuen ondorioz, gelatinan oinarritutako produktuen inplementazio kliniko segurua eta eraginkorra azkartu eta handitzea espero da.

6. ESKERRAK

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Erreferentzia zerrenda 33- 43 orrialdeetan aurkitzen da

Gelatinan oinarritutako terapeutikako azken aurrerapenak

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LABURPENA

Sarrera: Biomaterialek aukera zoragarriak eskaini izan dituzte ehunen ingeniaritza eta medikuntza birsortzailean. Gelatina, kolagenotik eratorritako biopolimero naturala, medikuntza birsortzaileko aplikazioetan oso erabilia izan da urteetan zehar, zelulentzat egokiak diren ezaugarriak aurkezten dituelako eta biomolekula sorta zabalaren askapena bideratzeko duen gaitasunagatik.

Landutako gaiak: Gelatinak biomaterial gisa aurkezten dituen propietate aipagarrienak eta bere aplikazio terapeutiko nagusienak azaltzen dira. Erabilera hauen artean, besteak beste, farmakoen askapenerako sistemak, ehunen ingeniaritzan egindako saiakerak, 3D/4D bioinprimaketarako balizko tintak edota *organ-on-a-chip* egiturak (laborategian prestatutako organu simulatzaile moduan definitu daitezkeen sistemak) biltzen dira.

Aditu iritzia: Polimeroen kimikan, mekanobiologian, irudien teknologian eta 3D biofabrikazio tekniketan emandako aurrerakuntzek gelatina ikerketa biomedikoko aplikazio anitzetan erabiltzea ahalbidetu dute, hala nola hezur eta kartilago ehunen ingeniaritzan, zaurien orbaintze prozesuetan eta minbiziaren aurkako terapietan. Berrikuspen labur honetan, gelatina biomaterialean oinarritutako formulazioekin farmakoen askapenean eta ehunen ingeniaritzan egindako azken aurrerapenak nabarmentzen dira. Gainera, material honen erabilerak aurkezten dituen erronka eta muga garrantzitsuenak ere aipatzen dira.

Hitz-gakoak: gelatina, ehunen ingeniaritza, medikuntza birsortzailea, biomateriala, farmakoen askapena, terapia zelularra.

1. SARRERA

Biomaterialek sistema biologikoekin elkarrekintzak dituzten substantzia mota ezberdinen talde zabala osatzen dute. Gainera, azken hamarkadetan zehar medikuntza birsortzailearen baitan aurrerapausoak egiteko aukera zabala eskaini dute. Alde batetik biologia, farmazia, kimika eta materialen zientzietan dugun ezagutza zabalduz doan heinean eta, bestalde, diziplina hauen artean emandako elkarrekintzen ondorioz, azkar ari da zabaltzen biomaterialen erabilera farmakoen askapen kontrolatu eta ehun ingeniaritzako esparruetan [1]. Biomaterial mota askoren artean, polimero bioaktiboek hainbat abantaila eskaintzen dituzte estatikoak eta estimulagarri-ezak diren materialekin alderatuz gero. Izan ere, hainbat prozesu biologikoetan eginkizun aktiboa izan dezakete, hala nola, farmakoen askapen eta prozesu biofarmazeutikoetan, zelulen patua kontrolatzean, organoide zelularren eraketan edota gorputzeko ehunen konponketa eta birsorkuntzan ere [2,3].

Biomaterialen jakintza arloak aurrerapen are eta garrantzitsuagoak lortu ditu hainbat eta hainbat arlo ezberdinetan emandako aurrerapausoen ondorioz, hala nola: kimikan, punta-puntako irudigintzako teknologietan, zelula iturri berrien aurkikuntzan, farmakoen askapenerako egitura optimizatuen garapenean, mekanobiologian (indar fisikoak seinale biokimiko bihurtzeko prozesuak) [4] eta hainbat biofabrikazio prozesuren garapenean, hala nola hiru dimentsiotako (*three-dimensional* edo 3D) bioinprimaketa [5] eta auto-mihiztadura (*self assembly*) teknologiak.

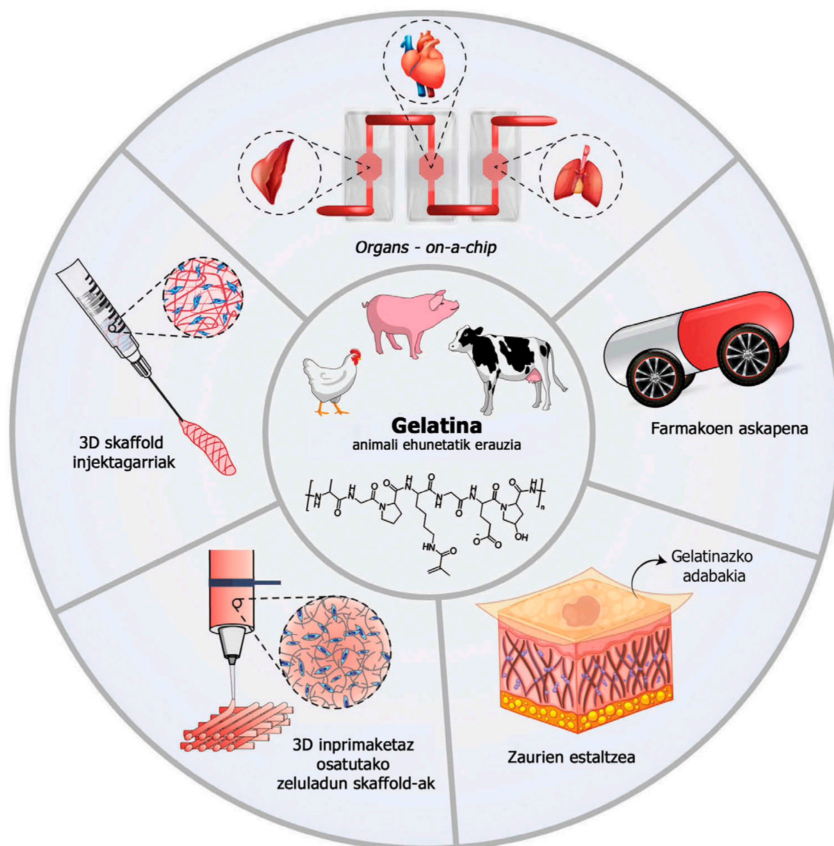
Aipatutako aplikazio biomediko horietan etorkizun handia izan dezakeen biomaterial emankorretako bat da gelatina. Kolagenotik erauzten den jatorri naturaleko polimeroa da gelatina, integrinak lotzen diren zelulen atxikimendurako peptidoak ditu bere egituran [6]. Gainera, metaloproteinasa (*matrix metalloproteinases* edo MMP) entzimei sentikorrak diren peptido sekuentziak dituenez, zelulek eragindako gelatinaren degradazioa gertatzea posible da. Bestalde, proteina hidrosolugarri honen ezaugarri nagusienetariko bat termo-sentikorra izaera da, soluzio tenperatura kritikoaren azpitik hoztean (25-35 °C) soluzio eta gel egoeren arteko trantsizio itzulkorra gertatzen delarik. Gaur egun material honek interes handia piztu du bai ehunen birsorkuntzara bideratutako skaffold-en garapenerako eta baita farmakoen askapen iraunkorra lortzeko eramaile gisa ere. Izan ere, zelulen bideragarritasun, hazkunde eta desberdintzapen prozesuak erregulatu ditzakeen biologikoki aktiboa den 3D mikroingurunea eskaintzen du. Gauzak horrela, hainbat dira mundu osoan zehar merkaturako diren gelatinan oinarritutako medikamentu eta produktu sanitarioak. Adibide moduan, Gelit-SPON®, Cutanplast®, Gelfoam® eta SurgiFoam® gelatinazko esponjak aipatu daitezke. Esponja xurgagarri hauek prozedura kirurgikoetan (neurokirurgia, toraxeko kirurgiak edo kirurgia oftalmikoak) tapoi hemostatiko moduan aritzeko onartu da. Honetaz gain, Estatu Batuetako farmako eta elikagaien administrazio (*Food and Drug Administration* edo FDA) erakundeak gelatina osagai moduan daraman hezur ehunaren ingeniaritzarako zuzendutako

alotransplantea kontsideratzen den produktua onartu du. DBX® Strip izenez ezagutzen den zinta osteoinduktibo malgu eta tolesgarri hau, hezur-matrize desmineralizatuz, gelatinaz eta sodio hialuronatoaz dago osatuta eta hezur hutsuneen betegarri gisa jarduteko dago onartua. Bestalde, gelatinaz osatutako XEN® Gel Stent inplante kirurgikoa glaukoma erregogorreko kasuetan begi-presioa modu seguru eta eraginkorrean murrizteko garatu da. Gelatina duten sendagaiak merkaturatzeari dagokionez, gizakietan erabiltzeko gelatinen ekoizleek segurtasun eta kalitate baldintza zorrotzak gainditu behar izaten dituzte. Kontrol zorrotz hauen helburu nagusia, animalia jatorriko lehengaien erabilerarekin lortutako behi-entzefalopatia espongiforme arriskua saihestea da. Aipatzekoa da, kalitate farmazeutikoko gelatinak jangarriak diren gelatinei dagozkien eskakizunak ez ezik, produktu medikoei dagozkien xedapenak ere bete behar dituzte. Ildo horretan, Europako Farmakopeak kalitate farmazeutikoko gelatinak ekoizteko beharrezkoak diren baldintzak zehatzak zerrendatzen ditu.

Berrikuspen honetan, gelatinak biomedikuntzan izan ditzakeen aplikazio potentzialak nabarmentzen dira, farmakoen askapenean eta ehunen ingeniaritzan material honekin egindako aurrerapenak deskribatuz.

2. GELATINAN OINARRITUTAKO BIOMATERIALAK

Zelulen gain eragiteko aurkezten duen gaitasuna dela eta, gelatina materiala oso erabilia izan da medikuntza birsortzailean eta ehunen ingeniaritzan. Gelatina jatorri naturaleko polimero bat da, bere aitzindaria den kolagenoaren aldean hainbat abantaila eskaintzen dituelarik. Helburu biomedikoetan erabiltzeko kolagenoaren muga nagusietako bat, baldintza neutroetan aurkezten duen ur-disolbagarritasun urria da [7]. Eragozpen nagusi hau, gelatina erauzteko prozesuarekin gainditu daiteke. Gelatinaren beste ezaugarri aipagarri bat, terapeutikoki aktiboak diren hezkunde faktoreekin, nukleotidoekin edota polisakaridoekin konplexu ionikoak sortzeko gaitasuna da [8]. Hori dela eta, biomolekula mota sorta zabalaren eramaile gisa jarduteko aproposa da (1. Irudia). Gelatina erauzteko erabilitako baldintzen arabera gelatinaren karga netoa positiboa (IEP= 9 , A motako gelatina) edota negatiboa (IEP=5, B motako gelatina) izan daiteke pH 7,4 denean. Horri esker, kontrako kargak dituzten molekulekin elkarrekintzak sortu eta euren bioaktibitatea mantentzea lortzen da. Honen ondorioz, gelatina farmakoen askapenerako prestatutako mikrokapsulen eta mikrosferen osagai moduan erabili izan da [9]. Gainera, material honen erauzketarako erabilitako iturri eta baldintzen arabera propietate fisiko-kimiko (urtze tenperatura, gel modulua edo biskositatea) ezberdinak dituzten gelatinak lortzen dira. Ezberdintasun hauen oinarria aminoazidoen proportzioa eta pisu molekularra izaten dira [10]. Bestalde, gelatina erraz funtzionalizatu daitekeenez, neurrira egindako ezaugarriak dituzten materialak sortu daitezke, aplikazio terapeutiko berrientzat aukerak sortuz.



1. Irudia. Gelatina biomaterial primario gisa dituzten garatutako egitura nagusiak: zaurien estalketarako adabakiak, hidrogel injektagarriak, 3D skaffold-ak, mikro eta nanopartikulen bidezko farmakoen askapena, biotintak eta organu-txipak.

Adibide gisa, zelulen proliferazioa eta desberdintzapena bideratzeko gelatinan oinarritutako 3D mikrogelak aipatu daitezke. Sistema hauek, lesio gunean ama-zelulekin kargatutako mikropartikulen eragin birsortzailea hobetu dezaketela ikusi da [11]. Gelatinazko elementu hauekin, zizaila-indarrek eragindako injektatutako zelulen heriotza murriztea lortu daiteke, xede den gunean zelulen atxikipena areagotzea ahalbidetzen duen ingurunea eskaintzen dutelarik.

Gelatinazko 3D skaffold erretikulatuak zelulen 3D kultiboa bideratzeko erabili daitezke. Hala ere, erretikulazioa termikoa den kasutan, sortutako hidrogelak hauskorrak eta ahulak izaten dira oro har. Arazo honi aurre egiteko, kobalenteki erretikulatutako gelatinazko hidrogelak ohiko prozedura kimikoak erabiliz lortu daitezke [12]. Entzimatikoki elkargurutzatutako hidrogelak sortzea hainbat ikerlarik proposaturiko estrategia izan da. Helburu

horretarako, askotariko entzimak erabili izan badira ere zaldi peroxidasa [13] edota transglutaminasa mikrobiarra [14] izan dira emaitza egokienak lortu dituzten entzimak. Sistema hauek ehunen ingeniaritzako helburu desberdinetarako diseinatu izan dira, gelifikazio tasa eta indar mekaniko aldakorrak dituzten hidrogelak sortzeko aukera eskaintzen baitute. Adibidez, entzimatikoki erretikulatuta dagoen gelatina-azido hidroxifenilpropioniko hidrogel konposatu injektagarria kartilago [15] eta garun [16] ehunen konponketan erabiltzeko ebaluatu dira. Horrez gain, argiarekin erretikulatu daitekeen gelatina metakrilato (GelMA) hidrogelek interes handia piztu dute hainbat aplikazio terapeutikoetan: kornea ehunaren ingeniaritza [17], nerbio periferikoen birsorkuntza [18] edota kartilago egituren prestakuntza [19]. Bestalde, aipatu beharra dago orain gutxi gelatina kobalenteki erretikulatuta duten hidrogel injektagarri berrien garapena, tetrazina eta norborneno talde funtzionalekin eraldatutako polimeroen arteko klik erreakzio kimikoan oinarritzen direnak hain zuzen ere [20]. Polimero hauek elkartze hutsarekin, elkargurutzaketa prozesua hasten da, *in vivo* injektatzen direnean degradagarriak izaten jarraitzen dutelarik. Gainera, zelulen bideragarritasun handia baimentzen dutela ikusi da, hidrogel hauen barnean kapsularatutako zelulek 3D morfologia luzatua garatzeko aukera dutela frogatuz.

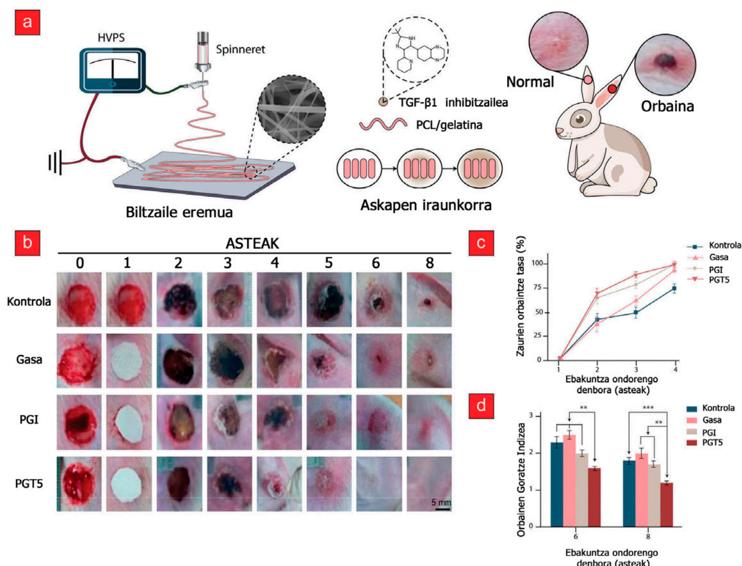
3D bioinprimaketa teknologian egindako etengabeko progresiboek, materialen, zelulen eta biomolekulen espazio-antolamendu gaineko kontrola areagotzea baimendu dute. Hori dela eta, badirudi jatorrizko ehunen 3D egitura intrintsekoa fabrikatzeko erraztasunak sortu direla. Azpimarratzeko adibide gisa, Jia W eta bere lankideek odo-hodien egitura fabrikatzeko garatutako geruza-anitzeko estrusio koaxiala inprimaketa estrategia aipatu daiteke. Egitura horien inprimaketa alginato sodikoa, gelatina metakrilatua eta polietilenglikol-akrilatu polimeroen nahasketan oinarritutako biotintekin burutu zuten. Erretikulazio bikoitza (kobalentea eta ionikoa) zuen inprimatutako 3D egitura honetan zelula endotelialak eta ama-zelulak barneratu zituzten, euren proliferazioa egokia izanik odol-hodien eraketa erraztu zela frogatuz [21].

3. FARMAKOEN ASKAPENEAN EGINDAKO AURRERAPENAK

Gelatinan oinarritutako biomaterialak oso interesgarriak dira farmakoen askapen sistemen diseinu eta fabrikaziorako. Sistema hauek, molekula bioaktiboen askapen kontrolatua, iraunkorra edota zuzendua bermatzen dute, farmakoen bioerabilgarritasuna eta efektu terapeutikoak hobetzen laguntzen dutelarik. Estrategia hau hainbat aplikazio biomedikoetarako erabiltzen da, hala nola ehunen birsorkuntza, zaurien sendaketa, minbiziaren tratamendua edota irudi medikoko tekniketan. Zaurien sendaketa eta orbaintzea prozesu dinamiko oso konplexua da, non hondatutako edo zauritutako azal ehuna konpondu edo ordezkatu egiten den. Larruazaleko zauriak gehienetan trauma, kirurgia edo erreduren ondorioz sortzen badira ere, diabetesa bezalako gaixotasunek zaurien sendatzeko denbora luzatzea

eragin dezakete [22]. Gelatinan oinarritutako adabakiekin zauriak estaltzeak infekzioetatik babesteko gaitasun handia ematen du eta zaurien sendaketa prozesua azkartu dezakete, propietate mekaniko eta degradagarriak neurri handi batean moldagarriak direlako. Gainera, biobateragarritasun perfil egokia erakutsi izan du gelatinak eta zaurien estalketarako oreka hidrofobiko/hidrofilikoa lortzen laguntzen du, biomolekulak modu egokian askatzea lortu daitekeelarik.

Adibide interesgarri gisa, gelatina eta poli(ε-kaprolatona) (*poly(ε-caprolactone*) edo PCL) konbinatuz orbainak saihesteko elektroirundako zuntzezko skaffold-a aipatu daiteke (2. Irudia) [23]. Ikuspegi horren bidez, fibroblastoen *in vitro* gehiegizko proliferazioa inhibitzea lortu zen eta untxi belarrietan eginiko *in vivo* azterketan orbain iraunkorren sorkuntza saihestu zezakeela frogatu zen. Orain gutxi burututako beste ikerketa batean, PCL/gelatina nanozuntz eta silikatozko partikula biozeramikoekin skaffold eroalea fabrikatzea lortu da [24]. Sistema horren nanozuntzez osatutako mikroegiturak zelula kanpoko matrizea mimetizatzen du eta silikato zeramiketatik gertatutako silizio ioien askapenarekin konbinatuz, diabetearen ondoriozko zaurien sendatzean eragin sinergikoa eta onuragarria lortu daitekeela ondorioztatu dute.



2. Irudia. Orbaintze hipertrofikoaren tratamendua elektroirundako gelatina zuntzezko skaffold-ekin. (A) TGF-β1 inhibitzailez kargaturiko PCL/gelatina zuntzezko skaffold-en garapenaren ilustrazio eskematikoa. (B) Zaurien ebaluazio makroskopikoa tratamendu talde desberdinetan (PGI: PCL/gelatina skaffold-a; PGT5: PCL/gelatina/TGF-β1 inhibitzaile-dun skaffold-a). PGT5 taldeak soilik erakutsi zuen zaurien irregulartasunik gabeko berregiturazio egokia. (C) Denbora ezberdinetan talde bakoitzeko zaurien orbaintze tasa. (D) Talde bakoitzeko orbainen goratze indizea, 6 eta 8 asteren ondoren. Esanahi estatistikoa: ** $p<0.01$; *** $p<0.001$. TGF-β1- *Transforming growth factor – β1*; PCL: *polycaprolactone*. [23] erreferentziatik baimenarekin moldatua. Copyright 2017 American Chemical Society.

Molekula bioaktibo eta farmakoen askapen zinetika, neurri handi batean, gelatina sareen degradazio tasa eta ur xurgatzearen araberakoa izaten da. Propietate horietaz baliatuz, antibiotikoak modu kontrolatuan askatuz gero, bakterio multi-erresistenteei aurre egiteko aukerak lortu daitezke [25,26]. Adibidez, gelatina-azido hidroxipropioniko hidrogelak propietate bakterizidak dituen H_2O_2 arekin batera eratu dituzte, ezaugarri antimikrobiarrak dituzten forma injektagarri edo lainoztagarriak prestatuz [26].

Gelatinan oinarritutako sistemek farmakoak askatzeko izan dezaketen beste aukerazko erabilera minbiziaren kontrako terapia izan daiteke. Izan ere, kasu horietan garrantzitsua izaten da medikamentuen bioerabilgarritasuna hobetzea eta askapen zuzendua bermatzea. Adibide modura, zelula dendritikoen eta adenobirus onkolitikoen administrazio konbinatu-ak tumore ingurunean immunitate-efektu antineoplasiko nabaria eragin dezakeela ikusi da. Hala ere, tumore gunean gertatutako farmakoen inhibizio azkarrak eraginkortasuna murriz-tu lezake. Erronka horri aurre egiteko, gelatinan oinarritutako biodegradagarriak diren for-mulazio injektagarriak garatu dira, zelula dendritiko eta adenobirus onkolotikoen askapena luzatzea lortu delarik [27].

Azkenik, gelatinan oinarritutako mikro eta nanopartikulak hazkuntza faktoreen askape-na bideratzeko gailentzen ari diren biomaterial moldagarri eta eraginkorrak dira. Ezaugarri horiei esker, medikuntzaren hainbat alorretan alternatiba terapeutiko bide berriak zabalt-zen ari dira, hala nola minbiziaren tratamenduan, garuneko iskemika ondorengo lesioen neuroprotekzioan [28] edota immunizazioan. Adibide gisa, Sabet S.-k eta lankideek gara-tutako gelatinazko nanopartikulak aipatu genitzake. Nanopartikula hauek hepatitis C-aren aurkako txerto ez birikoak garatzeko diseinatu dira, birus horren ez-egiturazko proteina 2 (*nonstructural protein 2* edo NS2)-aren genearekin konjugatuz, immunizitatea eragin deza-keen gene hori bakterio zeluletara transferitzea hobetzen dela frogatu baitute [29].

4. AURRERAPEN BERRIAK EHUN INGENIARITZAN

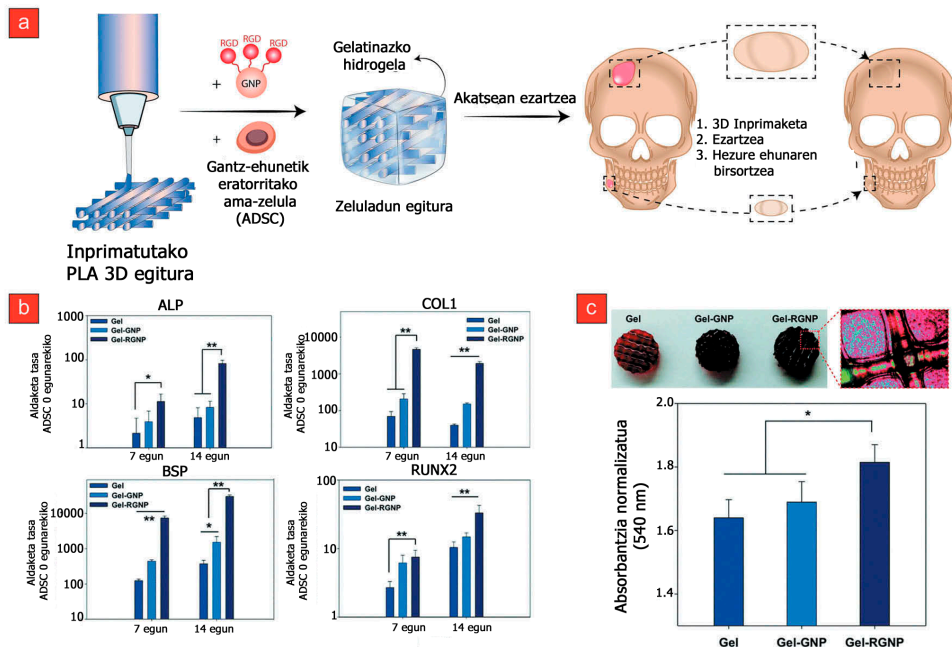
Ehunen ingeniaritzaren helburu nagusia, jatorrizko ehunen funtzio garrantzitsuenak bete ditzaketen sistema edo egitura artifizialen garapena da. Hala ere, helburu hori lortzea ez da gauza erraza, gorputzeko ehunek egitura oso konplexuak osatzen baitituzte eta sinergikoak diren osagai biologiko ugari aurkitzen dira bertan. Gaur egun, arlo horretan egiten ari diren aurrerapenen inguruan, ahalegin handiak ari dira egiten biomaterial mota ezberdinen kon-binaketaz jatorrizko efektu sinergikoak eta mikroegiturak lortzean. Ikuspegi honen bidez, gelatinan oinarritutako hainbat sistema garatu dira azken hamarkadan. Esate baterako, gelati-na eta azido hialuronikoaren konbinazioz sortutako hidrogelak ezaugarri elastiko aproposak izateaz gain, hainbat ehunen baskularizazioa bultzatzeko gaitasuna erakutsi du [30].

Elkartzen ez diren hezur hausturak epe luzeko ezintasuna eta mina eragiten dute. Ameriketako Estatu Batuetan soilik, urtero 8 milioi pertsona inguruk hezur-lesioak jasaten dituzte, eta ohiko tratamenduak ez dira eraginkorrak izaten kasuen %10 ean gutxienez. Hainbat ikerketa egin berri dira hezur ehunen ingeniaritzarako alternatiba eta eragile bioaktibo berriei buruz. Horren harira, gelatina-kitosanozko hidrogel konposatu batean plaketak barneratuz, arratoien hezur erradialaren akats ereduan hezur birsorkuntza areagotzea lortu zen [31]. Gainera, desberdintzapen osteogenikoarekin (fosfatasa alkalinoa, 1 motako kolagenoa, osteokaltzina, CD31 eta *Runx2* transkripzio faktorea) eta baskularizazioarekin (endotelio baskularraren hazkuntza faktorea) erlazionatutako hainbat generen RNAm-aren espresio mailak handiagotu zirela frogatu zen.

Gelatinan oinarritutako skaffold-ak hezurrak birsortzeko aplikazioetarako komertzializatu ez badira ere, gelatina fosfato kaltziko zeramikekin edota beste polimero sintetiko batzuekin konbinatzeak aplikazio klinikoetarako aukera oparoak eskaini ditzake [32].

Gelatina oinarritutako aldamioak oraindik hezurrak birsortzeko aplikazioetarako komertzializatu ez badira ere, gelatina kaltzio fosfato zeramikarekin eta beste polimero sintetikoekin konbinatzea aplikazio klinikoetarako aukera oparoak dira. Kasu hauetan, erronka handienetariko bat biomaterialak moldatuz hezur-akatsetan antzemandako arkitektura konplexuak sortzea da. Muga hori gainditzeko, hainbat estrategia ezberdin aztertu dira, besteak beste, sistema injektagarrien [33] garapena edota 3D inprimaketaz sortutako egitura inplantagarriak [34]. Estrategia hauek, pertsonalizatutako diseinua duten skaffold-ak fabrikatzea lagundu dezakete, hezur akatsean ezin hobeto moldatuz (3. Irudia).

Bestalde, kartilago ehunak modu espontaneoan konpontzeko duen gaitasun mugatua dela eta, ehun ingeniaritzak ikuspegi eta aukera berriak eskaintzen ditu ehun honen birsortzea bultzatzeko. Hainbat izan dira kartilago ehunaren ingeniaritzaren aplikazioetarako ikertu diren biomaterialak, gelatina besteak beste. Adibide bat aipatzearren, elektroirundako gelatina/PCL nanozuntzetan hezur-muineko estroma zelulak eta kondrozito zelulak elkarrekin kultibatuz, ama-zelulen kondrogenesia induzitu daitekeela ikusi da [35]. Bestalde, zelulez betetako gelatinazko hidrogelak ere kartilago ehunaren birsorkuntzara bideratu daitezkeela ikusi da. Kasu honetan, kondrozitoen aitzindari diren giltzadura-kartilagoko zelula multipotenteak zelula iturri interesgarri izan litezke [36]. Izan ere, kapsularatutako zelula hauek kartilago berriaren ekoizpenari dagokionez, desberdintzatutako kondrozitoek baino profil hobea aurkezten dute. Gainera, zelula hauetan PRG4 izenez ezagutzen den faktore lubrikatzailearen adierazpen maila altuak eta hipertrofiaren adierazle den X kolagenoaren adierazpen maila baxuek, zelula hauen birsorkuntza potentziala berretsi zuten.



3. Irudia. Hezur ehunaren birsorkuntza 3D inprimaketaren bitartez sortutako gelatinazko egiturekin, urrezko nanopartikula bioaktiboekin funtzionalizatu ondoren. (A) Hezur-ehunen birsorkuntza pertsonalizatua 3D inprimaketa bitartez lortzeko urratsen ilustrazio eskematikoa. (B) Desberdintzapen osteogenikoaren adierazle diren geneen espresio maila, ADSC zelulak gelatinazko hidrogeletan (Gel), urrezko nanopartikuletik funtzionalizatutako gelatina hidrogeletan (Gel-RGNP) edo RGD taldeekin konjugatutako urrezko nanopartikuladun gelatina hidrogeletan (Gel-RGNP) kapsularatu ondoren. (C) ADSC zelulak aurrez aipatutako hidrogeletan kultibatu ondorengo 21.egunean utzitako kaltzioaren tindaketaren irudi optikoak. Esanahi estatistikoa: * $p<0.05$, ** $p<0.01$. PLA: azido polilaktikoa; RGD: arginina-glizina-azido aspartikoa; ADSC: giza gantz-ehunetik eratorritako zelula-amak. [34] erreferentziatik The Royal Society of Chemistry-ren baimenerakin moldatua.

5. ETORKIZUNARI BEGIRA

Gelatina materialak medikuntza birsortzailean etorkizun oparoa duela aurreikusi daiteke, neurri handi batean biobateragarritasun profil egokiari esker eta bestalde ikuspegi biomediko berritzaileetan eskaini ditzakeen abantailengatik. Saiakera horien artean, zelulez kargaturiko gelatinan oinarritutako ehunen 3D modeloak aipatu genitzake, gaixotasunak hobeto ulertu edota farmako berrien garapena erraztu ditzaketen plataformak hain zuzen ere. 3D egitura mikrofisiologiko hauek gaur egun egiten diren *in vivo* ikerketak osatzeko edota ordezkatzeko balio ahal izango dute. Zentzu honetan, dagoeneko hainbat saiakera egin dira gelatinaz baliatuz. Adibide moduan, gelatinazko mikropartikulak pankreako tumore zelula eta

fibroblastoekin konbinatu dira, pankreako minbiziaren mikroingurunearen konplexutasuna aztertu eta estroma eta minbizi zelulen arteko erlazioak ikertzeko [37]. Bioingeniaritzako bestelako ikerketa bati dagokionez, zurruntasun eta degradazio tasa ezberdineko gelatinazko hidrogelak erabiliz, giza bihotzaren mikro-ehunak sortu dira. Kasu honetan, induzitutako giza zelula pluripotenteetatik eratorritako kardiomiozitoak izan dira gelatinazko hidrogeletan kapsularatu diren zelulak [38]. Bestalde, muskulu-eskeletikoa mimetizatzen duen 3D plataforma diseinatu berri dute zelulak kapsularatuta dituen gelatinazko hidrogelekin. Plataforma hauek, farmako eta kardiotoxinaren antzeko toxikoen hautatze frogetan erabiltzeko sortu dira [39], farmakoen aurkikuntza preklinikoetan eta garapenean garrantzitsuak izan daitezkeelarik.

6. ADITU IRITZIA

Gelatina, farmako eta proteinen askapena eta ehunen konponketa eta birsortzean propietate zirraragarriak dituen biomaterial ezaguna da. Gainera, egonkortasun mekaniko eta zurruntasun maila ezberdinak lortzeko erraz moldatu daitekeen materiala da, zelulen portaera eta desberdintzapenean eragina izanik, zelulen transplanteak egiteko ere erabilgarria izan daitekeelarik. Gelatina bakarka edota beste biomaterial batzuekin konbinatuta 3D/4D bioinprimaketarako biotinta moduan erabili daiteke, egitura anatomikoetara modu pertsonalizatuan egokitzen diren sistemak garatzeko aukerak eskainiz.

Hala ere, gelatinaren erabilpenak datozen urteetan zabaldu egin daitezkeela aurreikusten dugu. Material honen propietateek 2D eta 3D –tako nerbio egiturak garatzeko aproposak izan daitezke, gelatinan oinarritutako konposatu berriekin propietate mekaniko eta eroankortasun propietateak doituz. Gainera, aurrerapen hauek beste plataforma zelular batzuen garapena ahalbidetu dezakete; hala nola organu-txipak. Egitura berritzaile hauek, zelulen 3D kultibo eta kanal anitzeko mikrofluidikan oinarritzen dira, ehun eta organoen funtzioa ezagutzeko baliagarriak direlarik, baita farmakoek ehun horiengan izan dezaketen eraginak frogatzeko. Bestalde, gelatinan oinarritutako material konposatu eta desberdindutako giza ama-zelulekin egindako saiakerek itxaropentsuak diren emaitzak lortu dituzte bizkarrezur-muineko lesioen sendaketetan, bigarren mailako kalteak mugatzeko aukerak sortuz.

Gelatinak aurkezten duen beste erabilpen interesgarri bat, produktu terapeutikoak administrazio transdermikoaren bitartez ematean datza. Biobateragarriak, biodegradagarriak eta biosentikorrak diren gelatinan oinarritutako mikro-orratzak erabiliz, larruazalean zehar farmakoak modu seguruan emateko plataforma berriak ikertzen ari dira dagoeneko, kostu-eraginkortasun aspektuak kontuan hartuz betiere. Honetaz gain, gelatina autologoak diren hazkuntza faktoreekin arrakastaz konbinatzea lortu da, giza plasma eta plaketetatik eratorritako faktoreekin hain zuzen ere. Alderantzizko karga duten proteinak bahitu eta euren

bioaktibitatea mantentzeko duen gaitasuna dela eta, gelatinak fibrina skaffold naturalaren osagarriak diren propietateak eskaini ditzake, plaketetan aberatsa den plasmaren erabilpen terapeutikoaren potentziala areagotzea lortuz. Terapeutikarako estrategia hau, medikuntzako hainbat alorretan erabiltzen da jadanik, hala nola odontologia, ortopedia, dermatologia eta oftalmologian.

Hala ere, material interesgarri honen erabilgarritasunak hainbat erronka aurkezten ditu. Gelatinaren propietate mekaniko ahulak direla eta, zenbait aplikazio mugatuta egon litezke, batez ere eskakizun mekanikoak altuak direnetan. Horri aurre egiteko, sistema konposatuak eta gelatinaren egituran aldaketa kimikoak egitea aztertzen ari dira. Adibidez, gelatinaren egitura aminoazidikoa metakrilo, katekol, fenol eta epigalaktokatekin galato talde funtzionalekin aldatzea lortu da, propietate mekanikoak eta iraunkortasuna areagotzea lortuz.

Laburbilduz, gelatinak arlo terapeutiko anitzetan erabilera eta aplikazio ugari eskaintzen dituela esan daiteke. Polimeroen kimikan, biologia zelularrean, mekanobiologian, irudien teknologietan eta 3D biofabrikazio tekniketan izandako aurrerapenei esker, bere balioa eta potentziala zabaltzea lortu da bai farmakoen askapenean eta baita ehunen konponketa eta bisorkuntza alorrean ere.

7. ONDORIOA

Gelatinak eskaintzen dituen aplikazio aukerak oso ugariak dira, mikro eta nanopartikulen bidezko farmakoen askapenetik hasi eta biomimetikoak diren 3D skaffold-en bitartez bideratutako ehunen konpontze eta birsortzea adibidez. Arlo horietan eginiko ikerketetan egiten ari diren etengabeko aurrerapenei esker, gelatinan oinarritutako biomaterialak klinikara iritsi daitezkeela aurreikusten dugu.

8. ESKERRAK

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Erreferentzia zerrenda 56 - 57 orrialdeetan aurkitzen da

Hezurren birsorkuntzara bideratutako gelatinazko 3D formulazioen aurrerapausoak

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LABURPENA

Gaur egungo ehun-horniketa eta organo-emaileen eskasiak eta organoen transplante prozesuekin lotutako immunitate arazo larriek, zientzia diziplina berriak sustatzea ekarri dute, hala nola ehun ingeniaritza eta medikuntza birsortzailea. Azken hau, biologiaren eta ingeniaritzaren printzipioak uztartzen dituen diziplinarteko eremua da. Jakintza alor ezberdinetako kontzeptuak bateratuz, gaixotutako edota zauritutako ehunen birsortzea bultzatuko duten teknologien diseinua eta garapena du helburu, hala nola hezur-ehunaren birsorkuntza. Eginkizun horri aurre egiteko, hainbat eta hainbat estrategia garatu izan dira. Garrantzi berezia dute, ehun naturalaren birsorkuntza bultzatzen duten aldi baterako ordezkarien diseinurako biomaterialekin osatutako hiru dimentsiotako (*three dimensional* edo 3D) skaffold-ak, mikro zein nanopartikulak edota *in situ* erretikulatzen diren hidrogelen garapenak. Berrikuspen artikulu honetan, hezur-ehunen konponketa eta birsorkuntza sustatzeko balio duten formulazio horien diseinu eta fabrikazioan, gelatinak bete dezakeen zeregin nagusia azpimarratzen da. Medikuntza birsortzailearen baitako estrategietan, gelatinan oinarritutako formulazioen erabilpenaren ikuspegi orokor bat eskaintzen da. Inplementazio kliniko segurua eta eraginkorra lortzeko, gaur egun dirauten erronkak eta etorkizunerako aukerak eztabaidatzen dira.

Hitz-gakoak: gelatina, ehunen ingeniaritza, medikuntza birsortzailea, 3D skaffold-ak, farmakoen askapena, terapia zelularra.

1. SARRERA

Jakina da ehunen eta organoen transplanteak mugatuak direla gaur egun, emaile eskasiarengatik. Emaileak direnen kopurua urtetik urtera txikiagotu egiten den bitartean, organo baten transplantearen zain dauden gaixoen heriotza kopurua handituz doa. Ameriketako Estatu Batuetako Osasun eta Giza Zerbitzuen Sailak berriki jakinarazi duenez, 119000 pertsona baino gehiago sartu ziren transplanteren bat egiteko itxarote-zerrendan, baina 2016ko urtarriletik abuztura bitartean 10482 emaile soilik biltzea lortu zen [1]. Gainera, transplantea burutzea posible izaten den kasuetan ere, injertuarekiko sortu daitezkeen errefus arazoak kontuan izan behar dira, organo eta ehun-transplantea jasotako gaixoei zuzenean eragiten baitiete. Izan ere, gaixo horiek tratamendu immunoezabatzaileak jasotzen dituzte sarritan eta botika hauekin erlazionaturiko eragin desiragaitzak jasaten dituzte maiz. Gainera, gaur egungo medikuntzaren baitan, oro har, kaltetutako ehun osoa eta dagozkien funtzio guztiak ordezkatu ezin ditzaketen gailu biomediko eta protesiak erabiltzen dira. Hori dela eta, askotan gaixoaren osasun narriadura progresiboa saihestea ezinezkoa izaten da. Horretaz gain, herrialde garatuetan 65 urtetik gorako pazienteen ehunekoa pixkanaka handituz joango dela aurreikusten denez, medikuntzarako irtenbide berriak garatzea premiazkoa dirudi.

Medikuntza birsortzailea eta ehunen ingeniaritza medikuntzaren esparru berritzaileak dira, zeintzuak zaurituta edo akatsak dituzten ehun eta organoak ordezkatu edo leheneratzea duten helburu. Horretarako, funtzio anitz dituzten biomaterialez edota egitura biologikoez baliatzen dira [2,3]. Egitura horien garapenerako, jakintza-arlo ezberdinetatik eratorritako ezagutzak uztartzen dira, hala nola: biologia molekularra, biokimika, biomaterialen zientziak, bioingeniaritza, kimika, medikuntza eta teknologia farmazeutikoa. Hori dela eta, hondatutako edo galdutako ehun zehatz baten ordezkatzea edo berreskuratzea lortu nahi da, horretara bideratutako ordezko biologikoen diseinu eta fabrikazioaz. Teorikoki, kirurgia konpontzaile klasikoetan erabiltzen diren gailu eta inplanteekin alderatuz gero, egitura berritzaile hauek inguruko ehunetan integratzeko ahalbidea dute, kaltetutako ehunaren funtzioa erabat berreskuratzea lortuz eta tratamendu farmakologikoen beharra saihestuz [4].

Anbizio handiko helburu horri aurre egiteko, funtsezkoa da biomaterialez osatutako hiru dimentsiotako (*three dimensional* edo 3D) egiturak, seinaleztapen biologikoan diharduten molekulak eta, zenbait kasutan, zelulak ondo konbinatzea. Berrikuspen honetan, 3D skaffold-ek betetzen duten funtsezko papera aztertzen da. 3D egitura hauen funtzio nagusia aldi baterako euskarria izatean datza, hazten ari den ehunari laguntza kimikoa, fisikoa edota mekanikoa eskainiz. Jadanik deskribatu izan den moduan, eginkizun hori betetzeko, ezaugarri oso espezifikoak izan behar dituzte egitura horiek [5-7]. Ezaugarri horiei dagokienez, egitura osatzeko materialak biobateragarriak izan behar dira, propietate mekanikoak ere garrantzia dute, zelulen atxikipena, migrazioa, proliferazioa eta ezberdintzapen prozesuak ahalbidetuko dituen gainazala izatea komeni da eta egitura beraren forma ere kontuan eduki behar da.

Gainera, elkargurutzatutako sare porotsua izatea bilatzen da, osagai biologikoak bertan gordetzeko aukera izatea eta gainera biodegradagarria izan beharko du. Horretaz gain, degradazio prozesua nolabait kontrolagarria izatea komeni da, ehunen hazkuntza prozesuarekin bat etorriz. Bestalde, egituraren prestaketa erreproduzigarria izateak berebiziko garrantzia duela ikusi da. Ezaugarri interesgarri horietako hainbat 1.Taulan laburbildu dira.

1. Taula: Hezur-ehunen ingeniaritzarako biomaterialen propietate desiragarriak

Ezaugarriak	Ezaugarriaren deskribapena
Biobateragarritasuna	Ostalariaren hezur ehunaren birsorkuntza prozesuarekin bat egin behar du. Aldaketa kaltegarririk eragin gabe ostalariaren ehunekin harmonian aurkitzeko gaitasuna da.
Biodegradagarritasuna	Degradazio produktu arrotzik sortu gabe degradatu behar du materiala. Degradazio tasa ehunaren birsortze prozesuarekin egokitu behar da, birsortutako ehunak 3D egitura egokian antolatzeko aukera izan dezan.
Toxikotasun txikia eta hantura erantzun eza	Materialak ez du eragin toxikorik izan behar osteoblasto edota hezur-ehuneko gainontzeko zeluletan. Gainera, materialak ez dira toxikoak izan behar sistemikoki eta ez luke degradazio hondar toxikorik sorrarazi behar.
Porotasuna eta poroen arteko elkar-loturak	Hezur-ehunen ingeniaritzarako diseinatutako 3D egiturak porotsuak izan behar dira, elkar-lotutako poroak izanik. Poroen tamaina, bolumena, tamainaren banaketa, forma eta poroen hormen zimurtasunak zelulak bere baitan uniformeki banatu eta hazteko gaitasuna baldintzatuko dute. Parametro guzti hauek matrizearen baskularizazio gaitasunean eragina izango dute.
Egonkortasun biomekanikoa	Propietate mekanikoak (modulu elastikoa, trakzio-indarra, haustura gogortasuna edota luzatze portzentaia) ordezkatu edo birsortu beharreko hezurraren antzekoak izan behar dute.
Osagai bioaktiboen babesa eta askapen egonkorra	Biomaterialak agente terapeutiko potentzialen etengabeko eta kontrolatutako askapena bideratu beharko luke, hezurren birsorkuntza sustatzen duten hazkuntza faktoreen tokian-tokiko kontzentrazio egokiak lortzeko.
Prozesagarritasuna	Materiala erraz prozesatu ahal izatea komeni da, hainbat konfigurazio eta formulazio ezberdin diseinatu ahal izateko: 3D egiturak, partikula nano eta mikrometrikoak edota injektagarriak diren formulazioak.

Hezur-birsorkuntzari dagokionez, lesio jakin batzuetan hezurrak birsortzeko berezko gaitasuna badu ere, gehien transplantatzen diren ehunetako bat da [8]. Hezurren injertoa oro har, ehun transplanteen artean bigarren prozedura ohikoena da. Hauetako 2,2 milioi prozedura baino gehiago burutzen dira urtero mundu osoan zehar, odontologia eta kirurgia ortopedikoak oro har [9]. Aurretik azaldu moduan, adineko populazioaren hazkundeak hezur-haustura jasateko arrisku handiagoa duen biztanleriaren gorakada dakar. Bereziki, artrosia eta osteoporosi gaixotasunen prebalentziaren gorakadak, munduko osasun sistemetan eragin sozio-ekonomiko handiak izango dituela aurreikusten da [10].

Hainbat eta hainbat material eta fabrikazio teknologi ezberdin aztertuak izaten ari dira, hezur ehunaren birsorkuntzaren alternatiba gisa jarduteko helburuarekin. Gaur egun, hainbat dira hezur ehunaren ingeniaritzara bideratutako kolagenoan edota gelatinan oinarritzen diren produktu komertzial eskuragarriak, adibidez DBC® edota RegenOss® [11]. Berrikuspen honetan, gelatina biomaterial moduan erabiltzeko egindako aurrerapenak eztabaidatzen dira, batez ere gelatinan oinarritutako 3D egituren garapenean. Hori dela eta, orain arte lortutako emaitzarik esanguratsuenak eta baita aurretik dauden erronka mugatzaileak aztertu dira.

2. GELATINA BIOMATERIAL MODUAN

Gelatina kolagenoaren hidrolisi prozesutik eratorritako jatorri naturaleko proteina hidrosolugarria da. Hidrolisi prozesu horretan, kolagenoaren helize hirukoitz egitura apurtu eta harizpi bakarreko makromolekulak sortzen dira. Biomaterial honek portaera anfoterikoa erakusten du, basikoak eta azidoak diren aminoazido talde funtzionalak baititu bere egituran.

Hainbat urteetan zehar, gelatina erauzteko iturri ezberdinak erabili izan dira. Zentzu honetan, ugaztunetatik eratorritako gelatinak dira birsorkuntza prozesuetarako erabili izan diren ohikoenak. Gelatina hauek, nagusiki txerri eta behi azal eta hezurretatik erauziak izaten dira [12]. Hala ere, esan beharra dago, material hauek ez dituztela ehunen ingeniaritzan erabiltzeko beharrezkoak diren ezaugarri ideal guztiak erakusten. Izan ere, material hauen erabilerarekin, patogenoak izan daitezkeen bektoreen transmisio arriskua egon daitekeela deskribatu izan da noizbait; hala nola prioien transmisioa eta entzefalopatia espongiformearen garapena. Hori dela eta, arrisku hauek ekiditeko helburuarekin gelatinaren erauzketa lortzeko beste jatorri batzuk ikertu izan dira. Hainbat eta hainbat estrategia ezberdin erabiliz, ur hotz eta epeletako arrainen azal, hezur eta hegaletatik gelatina eskuratzea lortu izan da. Arrainetatik lortutako gelatinaren biskositatea, ugaztunetatik lortutakoena baino handiagoa da kasu gehienetan eta urtze tenperatura baxuagoa eta egonkortasun termiko txikiagoa izaten dute. Ezaugarri hauen ezberdintasunak, aminoazidoen konposizioan eta horien sekuentzian aldaketak daudelako izaten dira [13].

Animali jatorriko materialen desabantailak gainditu eta materialaren ezaugarriak hobetzeko helburuarekin, gelatina errekonbinanteak garatu izan dira. Pisu molekular eta puntu isoelektriko zehatzeko gelatinak hainbat adierazpen sistema ezberdin erabiliz sortu daitezke, hala nola: *Pichia pastoris* edo *Hansenula polymorpha* legamiak, *Escherichia coli* bakterioa eta sagu edo tabako landare transgenikoak. Teknologia horrekin, loteen arteko erreproduzigarritasuna nabarmen handitzen da, materialaren propietateen gaineko kontrola handitzen delarik. Oro har, bi estrategia ezberdin jarraitu daitezke gelatina birkonbinatua produzitzeko. Alde batetik, lehendabizi kolageno birkonbinatua sortu eta ondoren purifikatu eta desnaturalizatu egin daiteke kateen fragmentazioarekin edo gabe. Beste aukera bat, ordea, zuzenean gelatinaren kate espezifikoak sortzea da [14].

Gelatina aminoazido nahasketen polimeroa dela kontsideratu daiteke, 15 eta 400 kDa arteko pisu molekularra izaten duelarik. Biomaterial honen egiturari dagokionez, proportzio aldakorrean agertzen diren hogei aminoazido baina gehiagok osatzen dute lehen egitura. Gelatina molekularen baitan, glizina-X-Y hiruko egitura errepikakorrak agertzen dira, non prolina X posizioan eta hidroxiprolina Y posizioan aurkitzea den arruntena [15]. Kolagenoaren egitura tertziaroa hidrolisatzen denean, helize hirukoitz egitura bereizgarria hautsi eta polimero kate bakarrak (α-kateak), kobalenteki lotutako bi α-kate (β-kateak) edota hiru α-kateko egiturak

(γ-kateak) osatzen dira [16]. Kate polipeptidikoaren luzera eta kate mota bakoitzaren proportzioa hainbat aldagaien araberakoa izaten da: oinarrizko materiala, aurretratamendurako erabilitako metodoa, prozesuaren iraupena eta prozesua burutu den inguruaren hainbat parametro (tenperatura edota pH-a adibidez). Kolagenotik gelatina ekoizteko prozesua hiru fasetan banatu daiteke. Lehen urratsa, aukeratutako oinarrizko materialaren aurretratamendua izaten da. Ondorengo urratsa, erauzketa bere horretan izaten da eta azkenik erauzitako produktuaren purifikazio eta lehortze prozesuak [17]. Aurretratamendurako erabilitako metodoak, amaieran lortuko den gelatinaren karga elektrostatikoa zein izango den baldintzatuko du, bi motatako gelatinak bereizten direlarik. Kolagenoaren prozesu hidrolitikoa azido sulfurikoa edo azido klorhidrikoa erabiliz baldintza azidoetan burutzen bada, A motako gelatina lortuko da eta bere puntu isoelektrikoa 9,00 ingurukoa izango da. Aldiz, aurretratamendu alkalinoa egitea aukeratzen bada, lortutako gelatina B motakoa izango da eta 5,00 inguruko puntu isoelektrikoa aurkeztuko du. Peptido baten puntu isoelektrikoa, molekularen karga elektrikoa zero deneko pH-ari dagokio. Disoluzioaren pH balioa molekularen puntu isoelektrikoa baino txikiagoa denean, peptidoak positiboki kargatzen dira; aldiz, pH balioa puntu isoelektrikoa baino altuagoko baldintzetan negatiboki kargatuta egoten dira. Erauzketa prozesuaren parametroak aldatuz, puntu isoelektriko ezberdinetako gelatinak ekoizteko gaitasun hori, biomaterial honek esparru biomedikoan erabiltzeko aurkezten duen abantaila handienetariko bat da [18]. Hori dela eta, gelatina positiboki eta negatiboki kargatutako eragile terapeutikoen eramaile gisa erabili daiteke, ioien arteko konplexuak sortuz. B motako gelatina azidoa egokia da molekula basikoen askapena baldintza fisiologikoan bideratzeko eta A motako gelatina basikoa aldiz molekula azidoen garraioa burutzeko.

Gelatina jatorri naturaleko material biobateragarria eta ez-zitotoxikoa da eta kolagenoarekin alderatuz gero, immunogenizitate txikiagoa erakusten du. Polimero hau orokorrean seguru bezala onartua (*Generally Regarded as Safe* edo GRAS) dela kontsideratu du Estatu Batuetako farmako eta elikagaien administrazioak (*Food and Drug Administration* edo FDA) [19] eta gainera, biodegradagarria da, matrizearen proteinasa metalikoek (*matrix metalloproteinases* edo MMP) bideratutako degradazio entzimatikorako gune sentikorrak baititu egituran. Horretaz gain, degradazio entzimatikoaren ondorioz sortutako azpiproduktuak ere biobateragarriak direla ikusi da [20]. Gainera, gelatinaren egituran arginina-glizina-azido aspartiko (*arginine-glycine-aspartic acid* edo RGD) sekuentziak aurkitu daitezke. Sekuentzia horiek zelulen mintzean kokatzen diren integrinek ezagutzen dituzten gune espezifikoak dira eta zelula eta matrize extrazelularraren (*extracellular matrix* edo ECM) arteko lotura ahalbidetzen dute. Horregatik guztiagatik, zelulen atxikitzea bultzatzen duen peptido biomimetikoa dela kontsideratzen da, zelulak apoptositik babestu eta ehunen birsorkuntza bizkortzeko gaitasuna erakusten duelarik [21,22].

Gelatinaren propietate fisiko-kimikoei dagokienez, ezaugarri garrantzitsuenetariko bat uretan termikoki itzulgarria den sarea eratzeko gaitasuna da. Gelatinak konformazio aldaketa

aurkezten du 30 °C inguruko egoeran. Gelifikazio prozesuan zehar, tokian-tokiko zonalde ordenatuak sortzen dira, lotura ez espezifikoen bitartez elkartzen direlarik; lotura elektrostatikoak, hidrofobikoak edo hidrogeno zubi loturak. Aldiz, aurrez sortutako gel egitura berotzean, molekula arteko elkarrekintza horiek hautsi egiten dira, ezaugarri termo-itzulkorra lortzen delarik, gelatinaren propietate berezienetariko bat hain zuzen ere [23].

Gelatina dispertsioen propietateak definitzen dituzten hainbat faktore zehaztu dira. Hidrogelaren zurruntasuna, alde batetik, tenperaturaren araberakoa izango da. Hala ere, kontzentrazioak, berezko indarrak (*bloom* indarra), pH-a edo beste edozein gehigarriren presentziak dispertsio beraren gogortasuna aldatu dezake. Berezko indarra, hidrogela hozterakoan sortzen den egituraren indar intrintseko moduan definitu daiteke. Berezko indarraren balioa gelatinaren egiturak eta pisu molekularrak zehazten dute. Tenperaturaren eragina bereziki nabarmena da gel egituraren amaierako propietateengan; dispertsioaren biskositatea eta hidrogelaren indar balioak murriztu egiten dira tenperatura 40 °C-tik gorakoa denean [24]

Gelatinan oinarritutako formulazioak aplikazio medikoetan erabili ahal izateko, bereziki ehunen ingeniaritza esparruan, gelatina harizpien erretikulazioa edo elkargurutzaketa burutzea nahitaezkoa da, baldintza fisiologikoetan egonkortasun termikoa eta mekanikoa ziurtatzeko [25]. Azken urteetan hainbat ikerketa egin dira gelatina sarea erretikulatzeko erabilitako agente edo metodoek, amaierako egituraren propietate fisiko, mekaniko eta zitotoxikoetan dituzten eragina ikertzeko helburuarekin.

Gelatinaren egituran aldaketak eraginez gero, elkargurutzaketa prozesuaren gaineko kontrola hobetu daitekeela ikusi da, amaieran sortutako hidrogelaren propietateak hobetzea lortuz. Hori dela eta, norborneno, azido felurikoa, sukzinilo edo akrilamida bezalako talde funtzionalez baliatuz, ezaugarri bereziak dituzten formulazioak sortzea lortu da [26-29].

Gelatina hidrogelen elkargurutzaketa prozesua metodo fisikoak edota kimikoak erabiliz burutu daiteke. Gelatinaren elkargurutzaketa fisikoa lortzeko erabilitako prozesu fisikoen adibideak mikro-uhin energia [30], tratamendu dehidrotermala [31,32] edota erradiazio ultramorea [33] erabiliz eginikoak dira. Prozesu hauetan ez da biologikoki toxikoa izan daitekeen inolako konposatu kimikorik erabiltzen, baina esan beharra dago elkargurutzaketaren dentsitatearen gaineko kontrola edota prozesu beraren efizientzia askotan txikia izaten dela [34]. Aitzitik, gelatinaren talde funtzionalak aktibatzeko gaitasuna duten molekulak edota gelatinaren egituran tartekatzen diren konposatuak erabiltzen dira gelatinaren elkargurutzaketa kimikoa lortzeko. Aldehidoak, poliepoxidoak edota isozianatoak lotura kimikoak sortzen dituzte gelatina molekularekin, baina erreaktiboak edota toxikoak izan daitezkeen osagaiak askatzeko arriskua dago berauen degradazio prozesuan zehar [35-37].

Ildo horretatik jarraituz, aldehidoak izan dira gelatina erretikulatzeko gehien erabili izan diren konposatuak. Hala ere, era honetara elkargurutzatutako gelatinazko materialen

kasuan, degradazio produktuek zeluletan efektu toxikoak eragin ditzaketela frogatu da, immunogenizitatea eta hanturazko erantzunak nabarmentzen direlarik. Hori dela eta, eragin desiragaitz horiek saihesteko helburuarekin, azken urteotan beste hainbat alternatiba ikertzen ari dira.

Interes handia piztu duen gelatinaren elkargurutzaketa lortzeko erretikulazio eragileetako bat genipina da. Konposatu hau *Gardenia jasminoides* landareetatik erauzten den konposatu biobateragarria da, aplikazio biomedikoetan erabiltzeko hainbat material elkargurutzatzeko ikertzen ari direlarik. Konposatu honek erretikulazio eraginkortasun handia duela ikusi da eta glutaraldehidoarekin alderatuz gero 5000-1000 aldiz efektu zitotoxiko txikiagoa eragiteko gai dela frogatu da [38,39].

Baldintza fisiologikoetan egonkorrak diren gelatinazko egiturak lortzeko beste estrategia bat, entzimatikoki bideratutako molekula barneko loturak sortzea da. Tirosinasak, transferasak edota peroxidasen bidezko hidrogel erretikulatuak lortzea, ehunen ingeniaritzan interesa irabazten ari diren estrategiak dira. Erreakzio entzimatiko hauek inguru urtsuetan gertatzen dira, pH neutroan eta tenperatura moderatuetan. Gainera, substratuarekiko espezifikotasuna lortzea posible da [40,41]. Adibidez, perretxikoetatik eratorritako tirosinasa [42] edota transglutaminasa mikrobiarra (mTG) [43], gelatina elkargurutzatzeko erabili daitezkeen bi entzima dira.

3. GELATINAN OINARRITUTAKO SKAFFOLD-AK

3D skaffold-ak aldi baterako matrize extrazelular artifizialak dira, gorputzean zelula, farmakoak edota geneak administratzeko erabili daitezkeelarik. Egitura hauen funtzio nagusietako bat, zelulen hazkundea bideratzea izaten da. Izan ere, matrize hauek funtsezko zeregina dute zelulen 3D atxikimendu, proliferazio, desberdintzapen eta ehun berrien garapenean. Aurrez prestatutako skaffold-ak, teknika kirurgikoen bidez inplantatu daitezkeen ordezko egiturak dira. Fabrikatzeko erabilitako metodologiaren arabera 3D matrize porotsuak edota nanozuntzez osatutako matrizeak bereizi daitezke.

Azken urteotan, saiakera ugari egin dira hainbat helburuetara bideratutako gelatinan oinarritutako 3D skaffold-ak garatzeko: azalen birsorkuntza [44], zaurien orbaintzea [45] edota nerbio eta bihotz ehunen ingeniaritza. Hala ere, litekeena da orain arte egin diren aurrerapen esanguratsuenak, hezur-ehunen ingeniaritza alorrean lortu izana. Gelatinan oinarritutako eta beronekin sortutako hainbat konposite diseinatu dira hezur ehunak birsortzeko helburuarekin.

Hainbat produkzio teknika ezberdin erabili daitezke 3D skaffold-ak fabrikatzeko: disolbatzaile isurketa/partikulen lixibiatzea, liofilizazioa, fase banaketa, zuntzen lortura, fusiozko prozesaketa, elektroirutea edo ereduketa azkarra [46]. Gaur egun arte, gelatinazko matrizeak protokolo hauek jarraituz ekoiztu izan diren arren, prototipoen azken ezaugarriak hobetzeko

eta optimizatzeko metodologia berriak garatzeko esfortzu handiak egiten ari dira [47-49]. Adibidez, Jelen C. *et al.*-ek gelatina eta hidroxiapatita (*hydroxyapatite* edo HA) kontzentrazio aldakorrean dituzten geruzak elkar-pilatuz, funtzioen gradienteak dituzten skaffold-ak prestatu dituzte. Ezaugarri berri horren bidez, hezur ehunean porositate eta propietate mekanikoetan berez dagoen gradientea imitatzea lortu nahi da [50].

Hezurraren kasuan bezala, baldintza mekaniko gogorrak dituzten ehunetan erabiltzeko prototipoak diseinatzerakoan, hidrogelek berez aurkezten duten ur-eduki handia kontuan hartu behar da. Hori dela eta, formulazio mota horiek indar mekaniko mugatua erakutsi ohi dute. Oztopo hauek gainditzeko, gelatina bestelako konposatu ezberdinekin konbinatu daiteke, gelatinan oinarritutako skaffold konpositeen propietate mekanikoak hobetuz [51]. Adibidez, kaltzio fosfatozko zeramikak, bereziki interesgarriak izan daitezke hezur-ehunen ingeniaritzarako formulazioak diseinatzerako garaian, beharrezkoak diren propietate mekanikoak hobetzea ez ezik, ezaugarri osteogenikoak ere eskaini ditzaketelako. Konposatu horietako gehienek propietate osteokonduktiboak dituzte (osteoblasto zelulen atxikipena eta proliferazioa bideratzea), baina horietako batzuk gainera, propietate osteoinduktiboak (hezur berriaren eraketa bultzatzeko gaitasuna, zelula-amen errekrutatzea bultzatu eta leinu osteoblastikoetara bereiztea bideratuz) ere aurkezten dituzte [52,53]. Gaur egun, estrategia honen arabera diseinatutako sistemen adibide ugari aurkitu daitezke. Gelatina konpositeak HA [54-57], trikaltzio fosfatoa [58-60], kaltzio fosfato bifasikoa [61,62] edo oktakaltzio fosfatoa [63]-rekin osaturik, hezur-ehunen ingeniaritzan erabiltzeko nahasketa moduan proposatu izan dira.

Ezaugarri osteogenikoak dituzten gelatinazko matrizeek, hainbat motatako zelulekin dituzten elkarrekintzak ikertu izan dira, bai *in vitro* baita *in vivo* ere. Kasu askotan, mota honetako egiturek hainbat zelula mota, hezur-ehuneko zelula espezifikoetara ezberdintzatzeko gaitasuna dutela ikusi izan da. Ezaugarri horiek ebaluatzeko *in vitro* entseguen artean zelulen atxikipena eta proliferazioa, fosfatasa alkalinoaren (*alkaline phosphatase* edo ALP) aktibitatearen kuantifikazioa, hezurrarekin erlazionatutako geneen espresio azterketa edota tindatze immunohistokimiko espezifikoak nabarmendu daitezke. Zentzu horretan, sagu MC3T3-E1 osteoblasto zelula lerroa [64], giza osteoblasto primarioak [65], giza zelula-ama mesenkimalak (*human mesenchymal stem cells* edo hMSCs) [66] edota giza hortz-pulpako zelula-amak [67] emaitza onak lortu dituzten zelula mota batzuk baino ez dira.

Hala ere, zalantzarik gabe, egitura horiek hezur-ehuna birsortzeko duten gaitasuna ebaluatzeko, animalia eredu ezberdinetan egindako ikerketak oso garrantzitsuak dira. Arratoien garezurrean burututako tamaina kritikoko akatsen eredua, orain arte gehien erabili den metodoetako bat da [68-70]. Hala ere, bestelako animalia eredu batzuk ere aztertu izan dira gelatinazko skaffold-en garapenean: untxien kubitu hezur eredua [71], X izpiekin

irradiatutako animalia ereduak [72], arratoi femur kondilo eremua [73] edota arratoi tibia hezur eredua [74].

3.1 Gelatinazko skaffold-en bioinprimaketa

Zorionez, 3D bioinprimatze teknologiaren garapenarekin, gaur egun skaffold artifizialen egitura eta propietate mekanikoen diseinurako doitasun handiagoa lortzeko tresnak eskaintzen ditu. Teknologia aurreratu honek, ehunen ingeniaritzara bideratutako zehaztasun handiko prototipoen fabrikazioa erreproduzitzeko potentziala aurkezten du [75]. Teknika honetan, ordenagailuz lagunduta geruzaz-geruza materialaren deposizioa egitea lortzen da. Helburua ehunen ingeniaritzarako edota medikuntza birsortzailera bideratutako ehun eta organo bizien analogoak lortzea da, horretarako biomaterialez osatutako biotinten barnean zelula biziak edota bestelako konposatu biologikoak barneratzen direlarik [76].

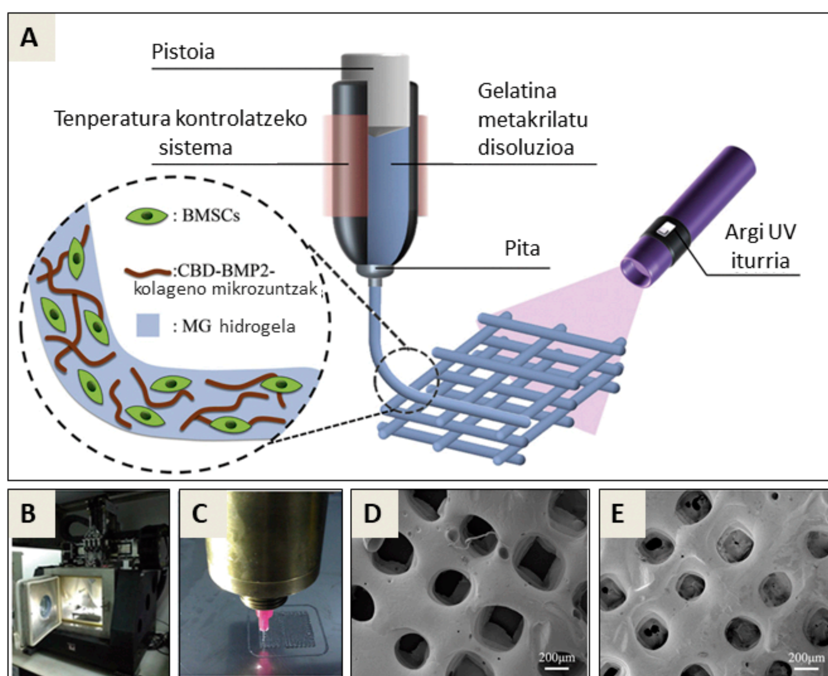
Teknika hau, oro har, bere garapeneko hasierako fasean dagoen arren, bioinprimaketa estrategiak etorkizun handia izango duela aurreikusten da, fabrikazio metodo aurrerakoia dela kontsideratzen baita. Bioinprimaketaren abantaila nagusien artean bereizmen handiko zelulen deposizioa, zelulen banaketarako zehaztasuna, kostu-eraginkortasuna eta eskalagarritasuna aipatu daitezke [77]. Azken ikerketetan frogatu izan den bezala, gelatina materiala biotintak garatzeko erabiltzean ezaugarri interesgarriak eskaini ahal ditu: biobateragarritasuna, biodegradagarritasuna, inprimatzeko gaitasuna, elkargurutzaketa prozesua burutu ahal izatea eta ezaugarri mekaniko egokiak. Gero eta gehiago dira material honek formulazio mota hauek garatzeko eskaini ditzaken abantailak aztertzen diharduten ikerketa taldeak [78-82].

Adibidez, 1. Irudian gelatina metakrilatu dispertsioarekin eginiko skaffold-en bioinprimaketa prozesua erakusten da. Ekipamentuak tenperatura kontrolatzeko sistema bat dauka eta gelatina elkargurutzatzeko prozesua argi ultramorearen bidez egiten da. Inprimagailu honek, matrizeen egitura eskala mikrometrikoan egokitzeko gaitasuna dauka, poroen tamaina doitu daitekeelarik [78].

3.2 Elektroirundako gelatinazko skaffold-ak

Azken urteetan, elektroirundako nanozuntzez osatutako sistemen garapena ikertu izan da. Izan ere, biomedikuntzako arlo ezberdinetan aplikatzeko erabilgarritasuna erakusten duen teknika da, hala nola, ehunen ingeniaritzarako ereduen garapenerako. Elektroirute teknikarekin 50 nm eta 10 μm arteko diametroa daukaten zuntzak sortu daitezke, horretarako jatorri naturaleko materialak edo baita sintetikoak ere erabili daitezkeelarik. Metodologia bakuna dela kontsideratzen da, non zenbait parametrok lortutako zuntzen morfologia eta tamaina definitzen duten. Prozedura honetaz baliatuz, ezaugarri nanometrikoak eta ECM naturala imitatzen duten elkargurutzatutako zuntzezko egitura biomimetikoak garatu izan dira [83]. Polimero ugari erabili izan dira, baita beraien arteko hainbat konbinazio ere. Hainbat

polimeroz osatutako zuntzak sortzeko, estrategia ezberdinak jarraitzeko aukera dago, hala nola: xiringa batean hainbat polimeroren dispertsio nahastea prestatzea, polimero bakoitza daraman xiringak aurrez-aurre kokatu prozesua aurrera eramateko edota dispertsio mota ezberdineko geruzatzea osatu [84]. Hainbat faktorek eragiten dute zuntzak izango dituen azken propietateengan. Hori dela eta, prozesuaren aldaerek aukera ematen dute ezaugarri ezberdinak dituzten zuntzen fabrikazioa burutzeko, nahi den erabilerara hobeto egokitzeko. Aipatutako faktore horiek guztiak hiru talde nagusitan sailkatu daitezke: Polimero dispertsioaren berezko propietateak (pisu molekularra, kontzentrazioa, disolbatzaile mota, biskositatea…), zuzenean prozesuarekin lotutako parametroak (eragindako potentzial-diferentzia, orratz eta gordailuaren arteko distantzia, orratzaren diametroa…) eta ingurumeneko parametroak (tenperatura, hezetasuna…) [85].



1. Irudia. 3D skaffold-en fabrikazioa 3D inprimagailu pertsonalizatua erabiliz. (A) Inprimaketa eta argi UV-az eginiko elkargurutzaketa prozesuaren irudi eskematikoa. (B) Hozkailuarekin hornitutako 3D inprimagailua. (C) Inprimaketa pita. (D,E) Gelatina metakrilatuzko inprimatutako skaffold-en behaketa mikroskopio elektronikoaz. (D) 363 ± 60 µm poro tamainako skaffold-ak eta (E) 282 ± 32 µm-tako poroak. Eskala: 200 µm. [78] erreferentziatik berrerabilia.

Teknika honen bidez gelatinazko zuntzak garatu ahal izateko, oso garrantzitsua da disolbatzailean gelatina molekularen konformazio egoera kontrolatzea. Izan ere, zuntz horiek sortzeko nahitaezkoa da gelatinak zorizko espiral edo "*random coil*" konformazioa izatea.

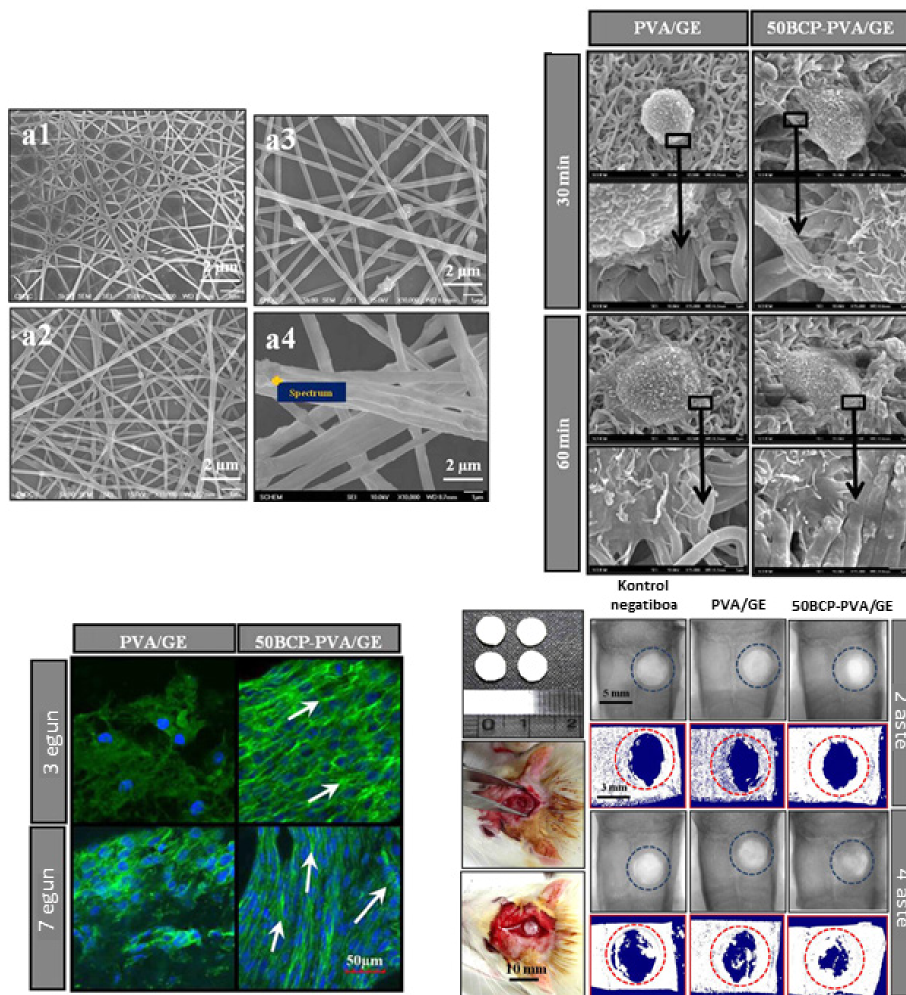
Gelatina uretan disolbatzen denean, beharrezkoa da nahasketa sol-gel trantsizio puntutik gora berotzea, biskositatea murriztu eta irutea burutzea posible izan dadin. Beste aukera bat, disolbatzaile organikoak erabiliz gelatinaren helize hirukoitz egitura ezegonkortzea da, gelatinazko elektroirundako nanozuntzak osatzeko aukera lortzen delarik. Estrategia hau erabiliz gelatinaren elektroirutea lortzeko, besteak beste, formamida, dimetilsulfoxidoa, azido azetikoa, azido formikoa, trifluoroetanola edota etilenglikol disolbatzaileak erabili izan dira [86].

Hezur-ehunera bideratutako egituren bioaktibitatea lortzeko helburuarekin, kaltzio fosfatoak edo beira bioaktiboak eransteak emaitza onak lortzea dakarrela ikusi da [87-89]. 2. Irudian ikus daitekeen moduan, kaltzio fosfato bifasikoz osatutako nanopartikulak polibinil alkohol eta gelatinazko elektroirundako zuntzetan gehitzeak, hezurrak *in vitro* eta *in vivo* konpontzeko gaitasuna duen materiala garatzea ekar dezake. Aipatutako nanopartikulak horien presentziak zuntzen diametroa handitzea eragiten du, tentsio indarra handiagoa lortzen da eta gainera, giza osteosarkomatik eratorritako MG-63 osteoblastoen atxikipena eta proliferazioa hobetzeaz gain, hezur formakuntzarekin erlazionatutako proteinen espresioa handitzea lortzen dela ikusi da. Horretaz aparte, arratoien garezurrean eginiko animalia entseguetan, hezurraren formakuntza handiagotzea eragin zen 2 eta 4 asteetan zehar [90].

Hezur-ehunen ingeniaritzarako formulazioen garapenean erabilitako bestelako estrategia, polimero eroale biobateragarrien erabileran datza, tokian-tokiko estimulu elektrikoak eman ahal izateko. 1950. urtean hezurraren berezko piezoelektrizitate propietatea antzeman zen, hezurraren berezko ezaugarri elektrikoa hain zuzen ere [91]. Beranduago aditzera eman zen moduan, ezaugarri honek eragin handia dauka osteoblastoen proliferazioaren kontrolean. Arrazoi hori medio, azken urteotan egindako hainbat ikerketetan antzeman den moduan estimulazio elektriko eta elektromagnetikoak hezur-ehunaren sendatzean eragin progresiboa izan dezake, osteoblastoen jardueran izan dezaketen eragina dela eta [92]. Elektroirute teknikarekin fabrikatutako gelatinazko matrize gero eta gehiagok aurkezten dituzte polimero eroale espezifikoak, hala nola anilina materiala [93]. Azken datuen arabera, polimero hau gehitzeak, alde batetik, egiturazko propietateen hobetzea dakar eta bestetik, hezur-sendaketa areagotzen du estimulazio elektrikoaren bitartez [94].

4. GELATINAZKO BESTELAKO 3D FORMULAZIOAK

Arestian aipatu moduan, gelatina materialaren abantaila interesgarrienetariko bat, formulazio mota ezberdinak garatzeko gaitasuna da. Erraz maneiatu eta prozesatzeko aukera ematen duenez, hautagai ezin hobea da ehun gogorren birsorkuntzara bideratutako 3D egitura eraginkorrak diseinatzeko. Hori dela eta, gelatina tamaina nano eta mikrometrikoko partikulak prestatu eta *in situ* gelifikatutako formulazioak sortzeko erabili izan da. Azken formulazio hauen abantaila nagusiena, inplantatua izateko beharrik gabe, zuzenean desiratutako lekuan injekzio bidez administratzeko aukera eskaintzen dutela da.



2. Irudia. Alkohol polibinilikoa eta gelatinazko (PVA/GE) nanozuntz biodegradagarriak hezurren birsorkuntzarako, kaltzio fosfato bifasiko (BCP) nanopartikulez funtzionalizatuta. (A) Morfologia azterketa, SEM-aren bitartez. a1-PVA/GE zuntzak; a2 – BCP %20 dituzten PVA/GE zuntzak; a3- BCP %40 dituzten PVA/GE zuntzak; a4 - BCP %50 dituzten PVA/GE zuntzak. (B) MG-63 giza osteoblastoen atxikipenaren azterketa elektroirundako mintzetan SEM irudiekin aztertuz, erein ondorengo 30 eta 60 minututan. (C) Osteoblastoak mintzen gainean kultibatu ondorengo mikroskopia konfokal azterketa, urdinez nukleoa eta berdez zitoeskeletoa antzemanez. (D) Arratoi garezur-akats ereduen micro-CT eta berreraikitako 3D irudiak, proposatutako formulazioak inplantatu ondorengo 2 eta 4 asteetara. [90] erreferentziatik baimenarekin egokitua.

4.1 Partikulak (Nanopartikulak eta mikropartikulak)

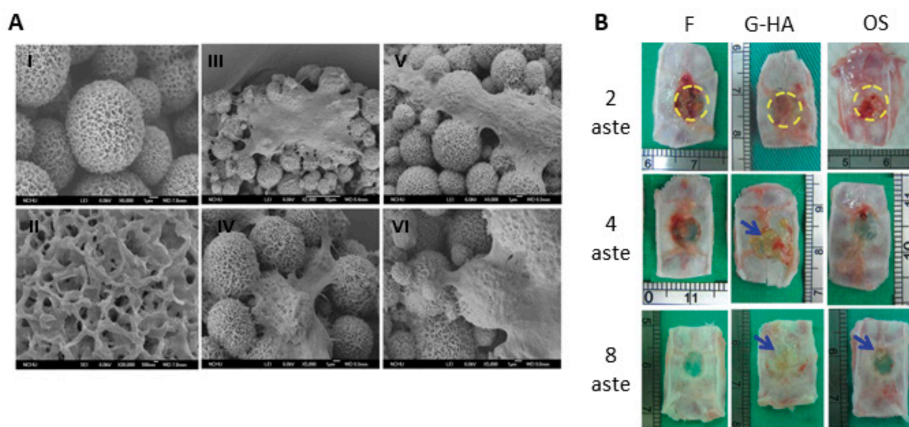
Gelatina partikulak osatzeko material moduan erabili izan da, hezur-ehunaren birsorkuntza hobetzeko estrategietan hainbat ezaugarri interesgarri eskainiz. Nano eta mikropartikulen diametro tamaina ezberdinak dira. Nanopartikula polimerikoek 1 eta 100 nm arteko neurria duten eramaile solido koloidalak kontsideratzen dira, aldiz, mikropartikulek hainbat mikratako diametroa izaten dute. Sistema horietan, fase oliotsua edo urtsua izan dezakeen barne-muina mintz polimeriko batez estalita agertzen da. Erdiko nukleoa sistema besikular baten bidez osatzen denean, kapsulak deritze (nanokapsulak edo mikrokapsulak). Aldiz, sistema matrizialei esferak deritze, non molekula bioaktiboak uniformeki dispertsatzea lortzen diren [95]. Mikropartikulen ezaugarririk garrantzitsuenetariko bat, aurkezten duten gainazal azalera handia da. Ezaugarri honi esker, barnean eraman ditzaketen zelulen bideragarritasuna handitzea lortzen da, elikagai eta hondakin-produktuen trukaketa baimenduta baitago. Hori dela eta, gisa horretako partikulek zelula-terapietarako eramaile moduan edota molekula bioaktibo handien askapenerako sistema moduan jarduteko aukera aurkezten dute [96].

Azken urteotan, ehun-ingeniaritza alorrean erabiltzeko gelatinazko partikulen diseinu eta fabrikaziorako hainbat metodologia garatu eta optimizatu izan dira. Nanopartikulak prestatzeko metodo ezagunenak bi urratseko desolbatazioa, koazerbazio bakuna, disolbatzailearen lurruntzea, mikroemultsioen prestaketa, nanoprezipitazioa eta auto-mihiztadura (*self-assembly*) teknikak izan dira [97].

Gelatinazko mikropartikulen ekoizpenari dagokionez ordea, gehien erabili izan den metodoa ur-olio emultsioen prestaketa eta jarraian eginiko elkargurutzaketa prozesuak dira. Hala ere, ekoizpen prozesu aurreratuagoak optimizatzeko ahalegin handiak egiten ari da ikerkuntza mundua, gainazaleko propietate hobeak dituzten formulazioak sortzeko, adibidez [98]. Sukzinilo taldeekin aldatutako A motako gelatinarekin egindako ikerlan batean, HA kristal mineralizatuak zituzten gelatinazko nanopartikulak sintetizatu zituzten, gelatinaren gainazalean gertatutako kaltzio ioien konplexazio prozesuari esker [29]. Gelatina eta HA-z osatutako material konpositeek emaitza onak lortu dituzte, osteobirsortze gaitasuna aztertzeko burututako ikerketetan [99]. 3. Irudian ikus daitekeen moduan, gelatina-HA-z osatutako 5-10 µm-tako mikropartikula esferiko uniformeekin Sprague-Dawley arratoien garezurrean eginiko entseguan, %90-eko hezur berriaren eraketa tasa lortu zen. Horretaz gain, giza osteoblastoen G-292 zelula lerroekin eginiko zitotoxikotasun entseguetan antzeman zen moduan, zelulen gain gutxieneko efektuak izan ditzakete partikula horiek, ehunen ingeniaritzarako ez direla formulazio toxikoak kontsideratuz [100].

Oro har, nahiko erraza da gelatinaren egituran talde bereziak barneratzea. Aukera horrek materiala funtzionalizatzea ahalbidetzen du, ehun zehatzetara zuzendutako formulazioak garatzea lortuz. Farbod K. *et al*,-ek gelatinazko nanopartikula biobateragarriak alendronato

konposatuarekin konjugatu zituzten, mineralizatutako ehunetara zuzendutako formulazioa garatuz. Izan ere, alendronatoan aurkitzen diren bifosfonato taldeek afinitate handia aurkezten dute hezur ehuneko mineral fasearekiko [101].



3. Irudia. Gelatina-hidroxiapatita (G-HA) konpositez osatutako mikroesferak ehun gogorren konponketarako. G-HA mikroesferen SEM irudiak (AI eta AII) eta osteoblasto zelulak mikroesferen gainazalean (AIII-AVI) 14 egunez kultibatu ondoren. (B) Garezurraren irudi digitalak, akatsean hainbat formulazio inplantatu eta 2, 4 eta 8 asteetara. F: Fibrin glue; G-HA: Gelatina-HA mikropartikulak; OS: Osteoset® Bone Graft Substitute. [100] erreferentziatik baimenarekin egokitua.

Hezur-birsorkuntzan erabiltzeko prototipoak diseinatzerako garaian, gelatinazko partikulen beste aplikazio desiragarri bat, egitura 3D esferiko horiek polimero sintetikoekin konbinatzea izan daiteke, material horien biodegradazio-profila hobetzea lortu baitaiteke. Zentzu horretan, polikaprolatonazko nanozuntzez osatutako skaffold-etan gelatinazko nanopartikulak sartzearekin degradazio eraginkorragoa lortu zen. Egitura horiek gorputzeko fluido simulatuan barneratzean, 7 µm luze zituzten nanozuntzak lortu ziren 8 asteetan. Gainera, nanopartikula horien presentziak skaffold-en poro tamaina handiagotzea eragin zuen, hMSC zelulen infiltrazioa hobetuz [102].

Gelatinazko mikropartikulak zelulen askapenerako sistema moduan erabiltzea terapia zelularren eraginkortasuna hobetzeko estrategia interesgarria dirudi. Gelatinazko mikropartikula hutsak zelulak barneratuta dituzten hidrogeletan gehitzean, partikula horien degradazio entzimatikoaren ondorioz zelulen atxikipenerako guneak sortu eta agente porogeno moduan jokatu dezakete [103].

Zenbait ikerlari gelatinazko mikropartikulek hMSC zelulen desberdintzapen osteogenikoan izan ditzaketen abantailak aztertzen ari dira [103-106]. Partikula mota huek zelula-amez osatutako esferoideetan barneratzean, nahiz eta zelulen antolamenduan eraginik ez izan, esferoideen propietate mekanikoetan eragina dutela ikusi da. Hain zuzen ere, mikropartikula horien presentziak zelulen mikroingurunea zurrunagoa izatea ahalbidetzen du, zelulen desberdintzapen ehun zurrunagoko zeluletara bideratuz, hala nola, hezur ehuneko zeluletara [105].

Antzerako beste ikerketa batean, gelatina metakrilatuzko mikropartikulak enbrioi ama-zelulen agregatuetan gehituz MMP aktibitatean eta zelulen desberdintzapenean induzitu ditzaketen aldaketak ikertu dira. Zelula hauetan, MMP entzimen espresio maila handiagotu egin zela nabaritu zen, ECMren birmoldaketa erraztea lortu eta zelulen desberdintzapena kontrolatu daitekeelarik. Emaitza hauen arabera, ama-zelulen morfogenesia eta desberdintzapena modulatu daitezke, ECMko materialez osatutako partikula degradagarriak erabiliz proteasen aktibitatea erregulatuz [106]. Aipatutako desberdintzapen prozesua kontrolatzeko beste estrategia bat, hazkuntza faktore zehatzekin kargatutako gelatinazko partikulak erabiltzea izan daiteke, zelula pluripotenteen agregatuetan gradiente morfogeniko zehatzak sortuz [107].

Zentzu honetan, partikula hauek ehunen ingeniaritza eta medikuntza birsortzailean eragile terapeutikoen ibilgailu gisa jarduteko ere aztertu izan dira. Izan ere, material honek aurkezten dituen propietate elektrostatiko eta degradazio proteolitikoak medio, biologikoki aktiboak diren faktoreen askapen iraunkorra duten formulazioak lortzeko material aproposa dela kontsideratzen da. Adibide gisa, hezur-proteina mofogenikoa – 2 (*bone morphogenetic protein-2* edo BMP-2) daramaten gelatinazko partikulak hezurren birsorkuntza sustatzeko eta azkartzeko duten potentziala aztertu izan da.

BMP-2 hazkuntza faktorea gelatinazko partikuletan kargatu eta ondoren *in vitro* askapen luzatua lortzen denean, proteinak bioaktiboa izaten jarraitzen du, hezur-eraketa nabarmenki areagotuz [108]. Askapenaren profilari dagokionez, hasieran bat-bateko askapen efektua lortzen da (*burst* efektua) eta ondoren lineala den zinetika. Zinetika honetan eragina duten zenbait faktore identifikatu izan dira. Askapenaren profilean dosiak efektu mugatua duela ikusi izan den arren, gelatina beraren izaera isoelektrikoak funtsezko zeregina duela ikusi da, materiala eta hazkuntza faktorearen artean sortzen den konplexazioa ionikoa izaten baita. Horretaz gain, partikulen elkargurutzaketa mailak ere zinetikan eragina izan dezake, faktorearen askapena neurri handi batean gelatinaren degradazio entzimatikoaren araberakoa izaten baita [109]. Proteina osteogeniko horren askapen iraunkorra lortzeko estrategia eraginkor bat, gelatina heparinarekin konbinatzea izan daiteke. Heparinak hazkuntza faktorea lotzeko eremuak eskaintzen ditu, beronen egonkortasuna areagotuz, desnaturalizazio eta degradazio proteolitikotik babestea lortuz [110]. Askapenaren gaineko kontrola hobetzeko beste aukera bat, metakrilo taldeekin aldarazitako gelatinarekin sortutako mikropartikulak osatzea da.

Aukera horretaz baliatuz, elektrostatikoki lotutako hazkuntza faktoreen askapen kontrolatua lortu daiteke ehunen ingeniaritzako proposamen berrietarako [111].

Alabaina, hainbat hazkuntza faktore askatzeko gai diren sistemak diseinatzeak, birsortze prozesuan eragiten duten tresna terapeutikoen eraginkortasuna hobetzea ekar lezake. Faktore ezberdinez kargatutako mikropartikula ezberdinak hidrogelez osatutako sistema batean sartzea, helburu hori lortzeko modu eraginkorra izan daiteke. Sistema huek faktore bioaktiboen askapena modu anizkoitzean gertatzea eragiten dute [112]. Adibide moduan, lotailu periodontaleko fibroblastoekin eginiko ikerketa batean, BMP-2 eta intsulinaren antzeko hazkuntza faktorea 1 (*insulin like growth factor* edo IGF-1) zituzten gelatinazko mikropartikulak edota bi mota horietako mikropartikulak skaffold makroporotsuetan barneratu zirenean, ALP aktibitatea handiagotzea eragin zen, kaltzio ioiaren deposizioa handitu egin zen eta zelulek osteokaltzina eta osteopontina gehiago produzitu zuten. Emaitza horiekin, bi mikropartikula mota horiek dituzten skaffold-ak aipatutako zelulen atxikipena, proliferazioa eta desberdintzapen osteoblastikoa bultzatzeko farmakoen eramaile funtzioa betetzeko oso erabilgarriak izan daitezkeela ondorioztatu zen, bi printzipio aktibo horiek modu sinergikoan aritzea lortzen baita [113]. Antzerako ikerketa lan batean, hasierako BMP-2 hazkuntza faktorearen askapena kitosanozko gel egituratik lortu eta ondoren IGF-1 hazkuntza faktorea gelatinazko mikropartikuletatik askatuz, zelulen jarduera osteoblastikoa areagotzea lortu zen [114]. Hainbat hazkuntza faktore dituzten sistema sinergiko dualak diseinatzerako garaian, kontuan hartu beharreko alderdi oso garrantzitsuak, faktore bakoitzaren dosia eta askatze denborak dira. Adibide gisa, BMP-2 eta fibroblastoen hazkuntza-faktore basikoarekin erabilitako dosi ratioen arabera, arratoien femur hezurraren kondilo distalaren osteogenesia bultzatzea edo oztopatzea lortu daiteke [73].

4.2 *In situ* sortutako hidrogelak

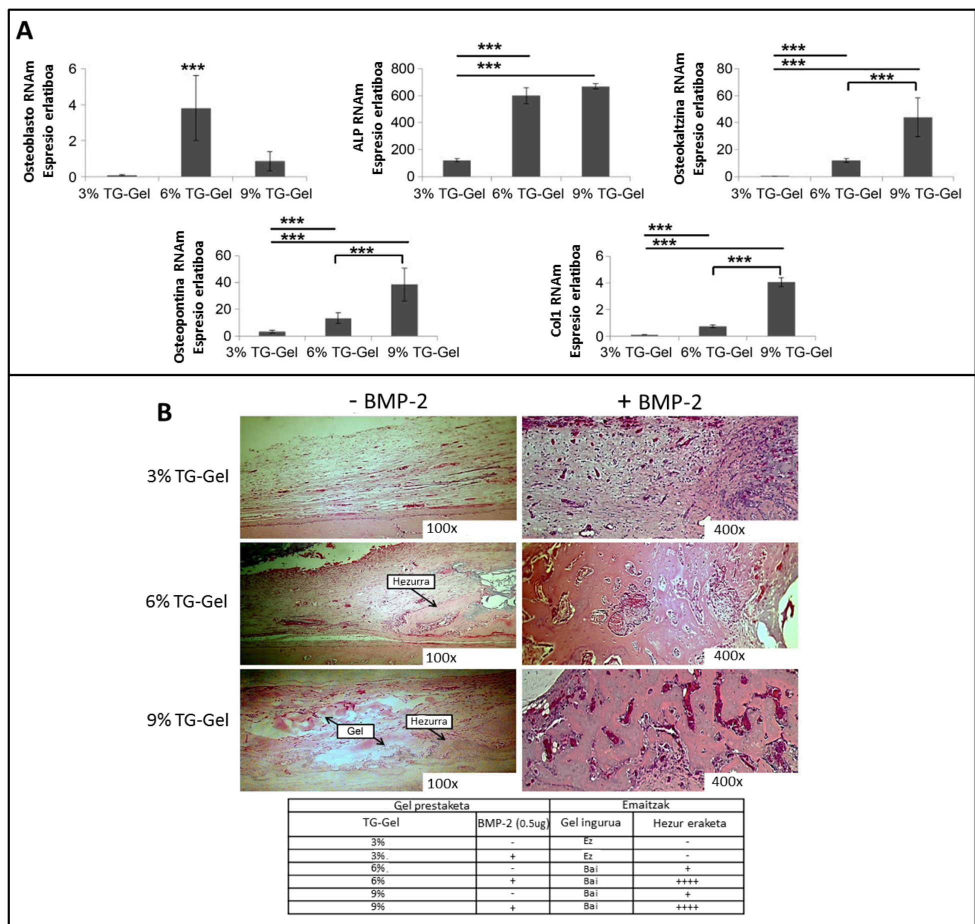
In situ gelifikatzen diren formulazioak injekzio bidez administratu daitezkeen ordezko moduan definitu daitezke. Administrazio hori guztiz inbaditzaileak ez diren eman bideak aprobetxatzen burutzen da, prozedura kirurgikoak egin beharra saihestuz. Ezaugarri honek ehunen ingeniaritzarako sistema eraginkorrak garatzeko indarrak bateratzen lagundu du, klinikara salto egin ahal izateko urrats bideragarria dirudielarik. Formulazio hauek, aurrez prestatutako 3D skaffold-ekin alderatuz gero, injektagarriak diren sistemen abantaila nagusienetariko bat forma irregularra duten hezurren akatsak tratatzeko baliagarriak suertatzen direla izan daiteke. Izan ere, formulazio hauek zuzenean lesio gunean injektatzen dira, bereizgarri egiten dituzten jariakortasunari esker. Mota hauetako sistemek hezur-ehunaren bereizgarri diren espazio-antolamendua erreproduzitzeko gaitasuna erakutsi behar dute, ortopediaren eta eremu kranio-fazialeko medikuntza birsortzaileetan hezurren betegarri gisa erabili ahal izateko [115, 116].

Jakina den moduan, hidrogelaren eraketa *in situ* baldintza fisiologikoetan gertatu behar da, modu azkar eta baldintza selektiboetan. Prototipo mota hauek diseinatzeko erabilitako metodologiei dagokienez, elkargurutzaketa kimikoak, elkarrekintza elestrostatikoak, auto-mihiztadura estrategiak edota estimulu-erantzun metodoak erabiltzeak lortu dituzte emaitza itxaropentsuenak [117]. Injekzio bidez administratzeko beharrezko propietateak lortzeko, gelatinan oinarritutako formulazioen optimizazioa burutzen dihardute gaur egun [118]. Zentzu horretan, "*Host-Guest Macromer*" deituriko estrategia batek emaitza onak erakutsi ditu, ezaugarri bio-itsaskorrak dituen hidrogela garatuz. Ezaugarri honetaz aparte, proposaturiko formulazioak konposatu hidrofobikoak gorde eta askatzeko gaitasuna duela erakutsi du. Makromero hau gelatinaren talde funtzional aromatikoen eta argia erabiliz erretikulatu daitezkeen akrilo taldedun β-ziklodextrinen konplexazioan datza. Kasu honetan, beharrezkoa da irradiazio ultramorea aplikatzea, gelatinaren harizpien polimerizazio fisikoa gertatu dadin [119].

Alabaina, Sandeep T. *et al.*-ek kanpoko energia estimulu, katalizatzaile edota polimerizazioaren haslerik behar ez duen gelatinazko hidrogel injektagarri bat diseinatu dute. Kasu honetan elkargurutzaketa toxikoak ez diren baldintzetan burutzen da. Hain zuzen ere, polimerizazioa gelatina egituran lotutako tetrazina eta norborneno talde funtzionalen arteko alderantzizkako Diels-Alder klik erreakzioaren bidez lortzen da [120]. Hidrogel hauek gainera, oso garrantzitsua den propietatea aurkezten dute: euren propietate mekanikoak aldakorrak dira eta ehun gogor zein bigunen birsortzea estimulatzeko egokitu daitezke [121].

Arestian aipatu moduan, entzimak erabiltzea oso interesgarria izan daiteke gelatina kateen elkargurutzaketan espezifikotasuna lortu nahi denean. mTG entzima erabiliaz sortutako gelatinazko hidrogelek ezaugarri mekaniko egokiak izan ditzakete hezur-ehunaren medikuntza birsortzailean behin-behineko substratu gisa erabili ahal izateko. Horretaz gain, efektu sinergikoak lortzeko aukera dago hazkuntza faktore zehatzak erabiliz gero (4. Irudia). Garezurreko lesioetan erabiltzeko formulazio osteokonduktiboak, entzima hau erretikulatzaile gisa erabiliz prestatu dira berriki [122,123].

Orain arte aipatutako estrategien ildo beretik, kaltzio osagaiak gelatinan oinarritutako formulazio injektagarrietan gehitzeak, hezur berriaren eraketa errazteko estrategia interesgarria dela dirudi [74]. Esate baterako, kaltzio fosfato hautsak, hidrogel injektagarrien propietate mekanikoak egokitu eta zelula amen desberdintzapen osteogenikoa bultzatzeko gaitasuna eskaini dezake. Orain gutxi, hidrogel-zeramikazko konposatu aurreratu bat hezur ehunetan aplikatzeko arrakastaz diseinatu da arrain jatorriko kaltzio fosfatoa, gelatina-3-(4-hidroxifenil)-azido propionikoa eta karboximetilzelulosa-tiraminaz osatutako hidrogel sistemarekin konbinatuz [124].



Gel prestaketa		Emaitzak	
TG-Gel	BMP-2 (0.5ug)	Gel ingurua	Hezur eraketa
3%	-	Ez	-
3%	+	Ez	-
6%	-	Bai	+
6%	+	Bai	++++
9%	-	Bai	+
9%	+	Bai	++++

4. Irudia. BMP-2 eta matrize zurruntasunaren eragina osteogenesian. Osteogenesiaren *in vitro* eta *in vivo* analisia *in situ* gelifikatzen den gelatina-transglutaminasa (TG-Gel) formulazioarekin. (A) TG-Gel hidrogelean kapsularatutako C2C12 zelulen bereizketa osteogenikoaren ebaluazioa transkripzio faktoreen mailen arabera. BMP-2 arekin hornitutako medioan hazitako zelulen emaitzak aurkezten dira, osagarririk ez zuten medioan hazitakoen RNAm-en adierazpenekin normalizatu ondoren. Emaitzak batez bestekoa ± desbideratze estandar moduan eta diferentzia estatistikoak (*p<0.05; **p<0.01; ***p<0.001) daude irudikatuta. (B) Hezur-eraketaren analisia TG-Gel formulazioak BMP-2 arekin kargaturik edo ez, larruazalpeko eman-bidetik dosifikatu eta 28 egun igaro ondoren. -: Hezurrik ez, +: 1-25% hezur, ++: 25-50% hezur, +++: 50-75% hezur, ++++: 75-100% hezur. [123] erreferentziatik baimenarekin egokitua.

5. AZKEN ONDORIOAK ETA ETORKIZUNERAKO IKUSPEGIA

Berrikuspen honek gelatina biomateriala hezur-ehunen konponketa eta birsorkuntzan erabiltzeko potentzialaren ikuspegi zehatza eskaintzen du. Gelatina, material nagusitzat duten formulazio mota posibleei dagokienez, hezur-akats handien kasutan, badirudi 3D skaffold porotsu eta nanozuntzezko sistema inplantagarriak egokienak izan daitezkeela. Bestalde, forma irregularreko akatsak birsortzeko, formulazio injektagarriek emaitza hobeak eskaini ditzakete. Formulazio hauen oinarrian, baldintza fisiologikoetan *in situ* polimerizatzen diren material erdi-likidoak daude. Aurrerapen handiak egin badira ere, biomaterial hau ohiko erabilera klinikora iristeko ahaleginak egiteke daude oraindik. Zalantzarik gabe, 3D bioinprimaketa teknikaren garapenak, prototipo irregularren diseinua aurrera eramaten lagunduko du. Horretarako, irudi teknikak erabiliz akatsaren azterketa burutzea oso baliagarria izan daiteke. Bestalde, segurtasunarekin eta erreproduzigarritasunarekin erlazionatutako araudietan egin beharreko aurrerapenekin, eta baita fabrikazio metodoen hobekuntzekin gelatinan oinarritutako formulazio aurreratu berritzaileak klinikara iristea lagunduko dute. Fabrikazio metodoei dagokienez, operatzaile gabeko teknologiak diseinatzea oso garrantzitsua dirudi. Teknologia hauetan, fabrikazio prozesuan zehar giza-manipulazioa murriztea lortu nahi da, errore esperimentalak gutxitu eta erreproduzigarritasuna hobetzea lortzeko helburuarekin.

6. ESKERRAK

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Erreferentzia zerrenda 77 - 83 orrialdeetan aurkitzen da

Helburuak

Gelatina kolagenotik eratorria den jatorri naturaleko polimeroa da, farmakoen askapenerako sistemak eta zeluletan oinarritutako terapietan euskarri funtzioa izan dezaketen hiru dimentsiotako egiturak diseinatu eta garatzeko aparteko ezaugarriak erakusten dituelarik. Material honek alderantzizko karga duten hazkuntza faktoreekin elkarrekintza ionikoak sortzeko gaitasuna du eta zelulen atxikimendurako talde funtzional baliagarriak aurkitzen dira bere egituran. Hori dela eta, hainbat ikuspegi terapeutikora bideratutako hautagai gisa ikertu da.

Nahiz eta ehun gogorretara bideratutako egiturak garatzeko hainbat muga aurkezten dituen biomaterial honek, gelatinak beste material batzuekin konbinatuz, konposatu funtzional aurreratuak sortzeko aukera zabala ematen du. Hori dela eta, material polimeriko hau, hezur-ehunen ingeniaritzarako hautagai oparoa izan daitekeela kontsideratzen da. Izan ere, material konposatuen azken ezaugarrien gain lortu daitekeen moldagarritasun handiaz baliatuz, gelatina materiala oinarri duten sistemak, kaltetutako hezurretara zuzenduak ez ezik, ehun honen inguruan aurkitzen diren trantsiziozko eskualdeetara ere bideratzeko aukera eskaini dezake. Trantsiziozko eskualde hauen bereizgarri dira konposizio, egitura eta ezaugarri biologikoekin osatzen diren gradienteak, tendoi-hezur interfazeetan aurkitzen diren moduan.

Hori guztia kontuan hartuta, Doktorego Tesi honen helburu nagusia, gelatinan oinarritutako plataforma polifazetikoa diseinatuz, hezur-ehunaren birsorkuntzara bideratutako hiru dimentsiotako sistemak garatzea izan da, tendoi-hezur interfazeen ingeniaritza barne harturik.

Helburu hori betetzeko, honako helburu espezifiko hauek finkatu dira:

1. Zelulen jarduerak sustatu eta hazkuntza faktoreak askatzeko gaitasuna duten entzimatikoki erretikulatutako gelatinazko hiru dimentsiotako skaffold-en garapena eta karakterizazioa.

2. Hidroxiapatita eta kaltzio sulfatoarekin sendotutako gelatinazko hiru dimentsiotako skaffold osteokonduktiboen garapena, karakterizazioa eta haien eraginkortasunaren *in vivo* ebaluazioa.

3. Ezaugarri anisotropoak dituzten gelatinan oinarritutako hidrogel konposatuen garapena eta karakterizazioa. Ehun-interfazeen ezaugarri estruktural eta konposizio ezberdinak gelatinazko hidrogel bifasiko bakarrean integratzea.

Atal esperimentala

1. KAPITULUA

Hezur-ehunen ingeniaritzara bideratutako entzimatikoki elkargurutzatutako gelatinazko 3D matrizeak

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LABURPENA

Hezur-ehunaren ingeniaritza azken urteotan garatzen ari den medikuntzaren alorra da, zeinak hezurraren birsortze gaitasuna gutxituta dituzten patologiak bideratzen dituen. Lan honetan, transglutaminasa mikrobiarrarekin elkargurutzatutako gelatinazko skaffold-en garapena eta karakterizazioa burutu da. Matrize hauek, aldi berean zelulen euskarri eta hazkuntza faktoreen askapen sistema moduan jarduteko gaitasuna erakusten dute. Zehazki, ezaugarri morfologikoak, biomekanikoak eta biologikoak ikertu dira. 3D sistemak prestatzeko garaian erabilitako entzima eta gelatina proportzioak eragin zuzena duela ikusi da bai matrizeek ur hartzeko duten gaitasunean eta baita propietate mekanikoetan ere. Burututako biobateragarritasun entseguen arabera, garatutako sistemak ez dira zitotoxikoak. Gainera, aurrez aukeratutako formulazioen jarduera biologikoa aztertu da L-929 fibroblastoak, D1 zelula-ama mesenkimalak (*mesenchymal stem cells* edo MSC) eta MG63 osteoblastoak erabiliz. Entsegu horien arabera, skaffold-ek MSC-en desberdintzapena eta seinaleztapena baimentzeko gaitasuna erakusten dute. Izan ere, matrizeen gainazalean ereindako zelulen perfil proliferatiboa eta *stemness* txikiagotu egin ziren, osteoblastoekin erlazionatutako geneen (*Col1a1, Runx2, Osx*) espresioa handitu zen heinean. Horretaz gain, matrizeetatik bideratutako endotelio baskularraren hazkuntza faktorea (VEGF) eta hezur-proteina morfogenikoa 2-aren (BMP-2) askapen kinetikak aztertu dira. Bi kasuetan, lehen mailako kinetika jarraituazaskatzen direla ikusi da. Emaitza hauen arabera, lan honetan garatutako matrizeak egokiak izan litezke hezur-ehunak birsortzeko helburuetarako.

2. KAPITULUA

Hezur-proteina morfogenikoa-2 (BMP-2) -arekin kargaturiko gelatina/biozeramika konposite bioinspiratuek hezur osteoporotikoaren konponketa sustatzen dute

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LABURPENA

Osteoporotikoa den hezur ehunaren gaitasun osteogeniko mugatua dela eta, bereziki zaila egiten da hezur hauen hausturen sendaketa prozesua. Hezur-proteina morfogenikoa-2 (*bone morphogenetic protein-2* edo BMP-2)-aren dosiak murriztea ahalbidetzeko, bioaktiboak eta osteonkonduktiboak diren biomaterialen garapena, lesio horien birsortze terapiak optimizatzeko estrategia itxaropentsu gisa kontsideratzen da. Lan honetan, hezur osteoporotikoa birsortzeko egokiak diren gelatinan oinarritutako hiru dimentsiotako skaffold osteokonduktiboak garatu ziren, kaltzio sulfatoarekin eta hidroxiapatitarekin sendotuz. Konposatu organiko/ez-organikoz osaturiko sistemek ura hartzeko ahalmen bikaina eta *in vitro* degradagarritasun ona erakutsi zuten. Skaffold-etan konposatu zeramikoak barneratzeak sistemen gogortasuna areagotzea eragin zuen, zelulen biobateragarritasunean eragin kaltegarririk antzeman gabe. Giza hezur-muineko zelula-ama mesenkimalekin eginiko entsegu biologikoek agerian utzi zuten garatutako hiru dimentsiotako egituren gainazala egokia dela zelulen atxikimendua eta proliferazioa bideratzeaz gain, desberdintzapen osteogenikoa bultzatzeko ere. Zehazki, zelula-amen adierazgarri diren (*Nanog, Oct4*) hainbat generen espresio maila txikiagotu zen heinean, desberdintzapen osteogenikoarekin erlazionaturiko hainbat markatzaileren (*ALP, Col1a1, Fmod*) mailak handitu zirela antzeman zen, oinarrizko baldintzetan eginiko entseguetan. Gainera, indartutako skaffold porotsuetatik BMP-2-aren askapena iraunkorra izatea lortu zenez *in vitro* eginiko entseguan, sagu osteoporotikoen garezur-gangako tamaina kritikoko akatsen eredua aukeratu zen prestatutako formulazioen gaitasun terapeutikoa aztertzeko. Aipatzekoa da animalia bakoitzean administratutako BMP-2 dosia erlatiboki txikia izan zela; 600 ng hain zuzen ere. Saguekin eginiko *in vivo* azterketen emaitzek agerian utzi zuten BMP-2-arekin funtzionalizatutako gelatina konpositeen skaffold-ek hezur eraketa nabarmenki handitzea lortu dezaketela 8 asteren buruan. Oro har, ikerketa lan honek gelatina/kaltzio biozeramika konpositeek hazkuntza faktore osteogenikoen eramaile gisa izan dezaketen potentziala erakusten du, hezur osteoporotikoen akatsen tratamendurako bereziki interesgarriak izanik.

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3. KAPITULUA

Ehun-interfazeen ingeniaritzara bideratutako ezaugarri mineralizatuak eta anisotropoak biltzen dituzten hidrogel bifasikoak

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LABURPENA

Ehun muskuloeskeletikoen interfazeen berezkoak diren egitura eta konposizio gradienteak eten eta ehun fibrotikoz ordezkatzen dira gaixotasun eta endekapen testuinguruan. Elkargune horietan aurkitzen diren trantsizio konplexuen lehengoratzea bultzatzeko ehun ingeniaritzako estrategiek garrantzi berezia dute medikuntza birsortzailearen baitan. Lan honetan, gelatinan oinarritutako fase anitzeko hidrogel sistema bat garatu da, non konposizio eta mikroegitura ezberdinak dituzten zatiak, unitate bakarrean intregatu diren. Helburu horretarako, gelatina sarea entzimatikoki elkargurutzatu da eta hidroxiapatita (*hydroxyapatite* edo HA) partikulak eta zelulosazko nanokristalak (*cellulose nanocrystals* edo CNC) erabili dira funtzio zehatzak egokitzeko. Alde batetik, partikula mineralizatuak formulazioan barneratuz, hidrogelen gogortasuna areagotzea lortu da eta bestalde, CNCen lerrokatze magnetikoa eraginez egitura anisotropoak prestatu dira. Giza gantz-ehunetik eratorritako zelula amekin egindako entseguetan agerian geratu den moduan, alde anisotropoan kapsularatutako zelulen hazkuntza lerrokatua izan da eta tenaszinaren sintesi eta deposizioa handiagoa izan da fase honetan. Bestalde, zelulak mineralizatutako zatian kapsularatu ondoren, jariatutako fosfatasa alkalinoaren aktibitatea eta osteopontinaren espresioa induzitu egiten dela antzeman da. Hori dela eta, tendoi eta hezur ehunekin erlazionatutako ezaugarriak bateratu ahal izan dira, fisikoki ezberdindutako eskualdeen mihiztaduraz hidrogel bifasikoak garatuz. Emaitzak hauek gelatina-transglutaminasa entzima tandemak, ehun konektiboen elkargune gradual, konposatu eta konplexuak imitatzen dituzten estrategien garapenerako eskaintzen duen moldakortasun potentziala nabarmentzen dute.

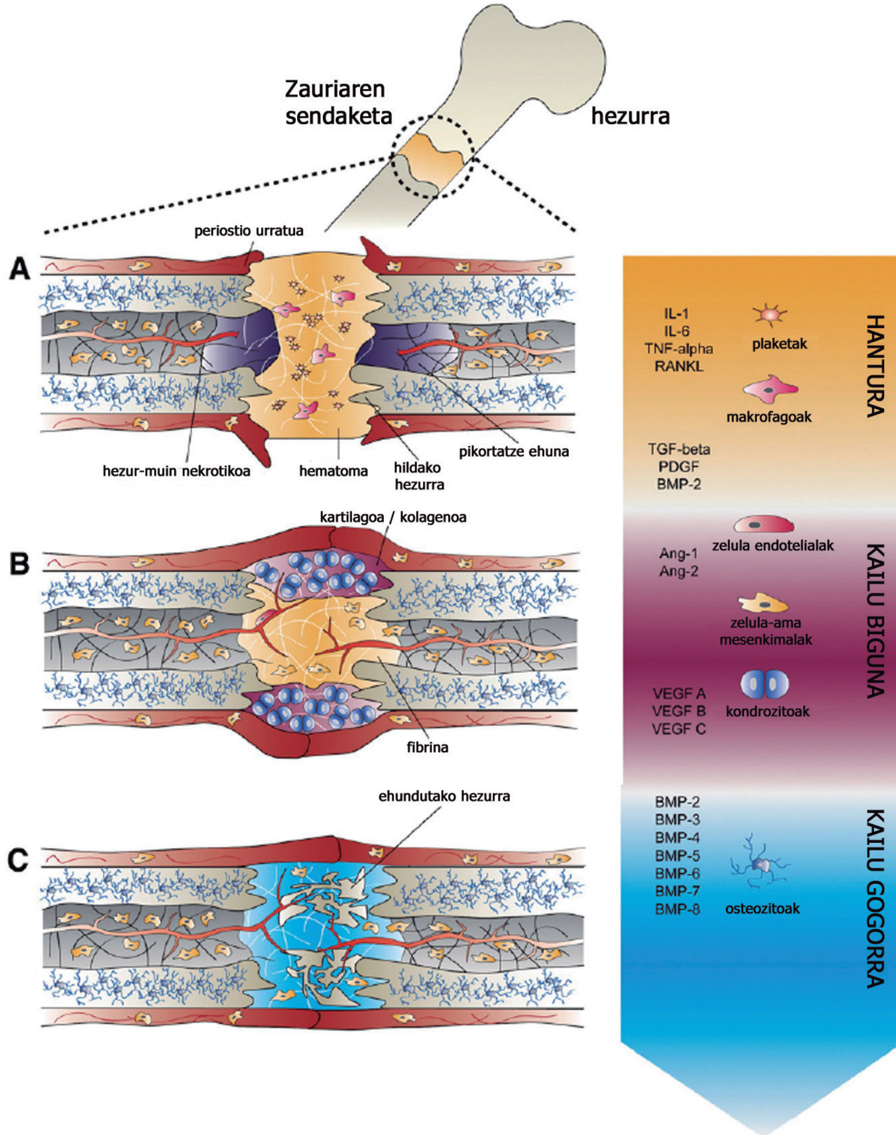
Eztabaida

Munduko Osasun Erakundearen arabera, baldintza muskulu-eskeletikoak sistema lokomotoreari eragiten dieten hainbat diagnostikok osatzen dute, mundu mailan ezintasun garrantzitsuenetarikoen kausak izanik [1]. Sistema lokomotorea hezurrek, muskuluek, giltzadurek eta hauekin erlazionaturiko hainbat ehunek, hala nola tendoiak eta lotailuak, osatzen dute, gorputzari euskarria, egonkortasuna eta aldi berean mugimendua eskaintzen dizkiotelarik. Gizartean eragin handia duten ezgaitasun baldintza ohikoak hezur-hausturak eta giltzadurekin erlazionaturiko patologiak dira. Tendoi eta lotailuetan gertaturiko lesioek sistema muskulu-eskeletikoarekin erlazionaturiko kontsulta medikoen %30-a osatzen dute, urtero mundu osoan 4 milioi gertakari berri antzematen direlarik [2]. Bestalde, osteoporosiak soilik mundu osoan zehar urtero 8,9 milioi hezur-haustura baino gehiago eragiten dituela estimatzen da [3]. Gainera, aipatutako arazo hauek gizabanakoengan eta gizarte osoarengan duten eragina nabarmen handituko dela aurreikusten da, munduko biztanleriaren zahartzearekin batera. Horrela, 2050. urterako, gizonezkoetan eta emakumezkoetan gertatutako aldaka hausturen mundu mailako intzidentzia %310 eta %240 igoko dela aurreikusi da, hurrenez hurren, 1990. urteko tasekin alderatuz [4].

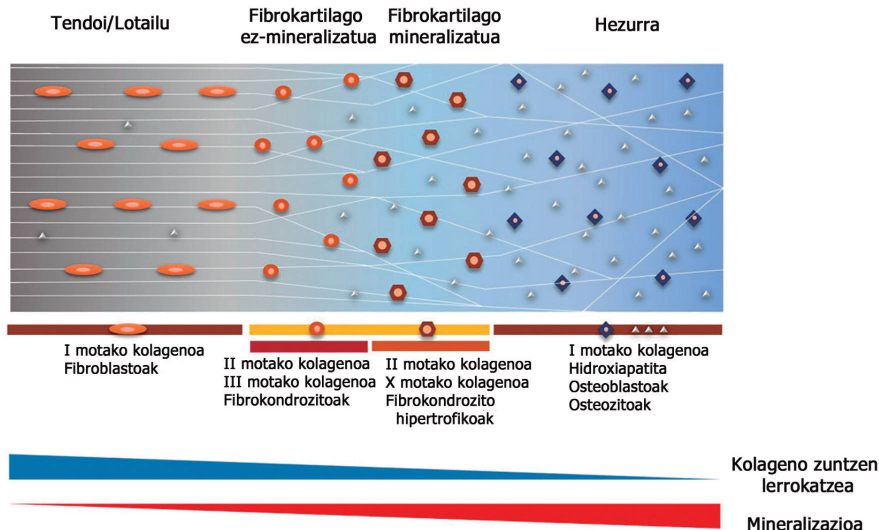
Helduaroko hezur-ehunak haustura bat pairatzen duenean, erabat birsortzeko berezko gaitasuna dauka. Horregatik, hezur lesio bat agertzen denean, osteogenesi mekanismoak berehala hasten dira martxan, lesioa gertatutako eremuan hezur-ehuna berreskuratzeko. Konponketa prozesu horren helburua jatorrizko geometria, matrizearen biologia eta ezaugarri biomekanikoak leheneratzea da [5]. Birsorkuntza hori lortzeko, angiogenesi eta osteogenesi prozesuen inplikazioa beharrezkoa izaten da, hainbat zitokina, hazkuntza faktore, hormona, zelula eta matrize estrazelularreko (*extracellular matrix* edo ECM) osagaiek estimulazioa bideratzen dutelarik (1. Irudia) [6]. Alabaina, badira hainbat egoera non birsorkuntzarako gaitasuna ez den nahikoa ehuna bere osotasunean sendatzeko. Azken estimazioen arabera, hezur hausturen %10 ez da gai bere kabuz guztiz osatzeko [7]. Kasu askotan, osteoporosi edo osteonekrosi bezalako baldintza patologikoek eragiten dute hezurra guztiz birsortzeko ezintasuna. Gaur egun, hezur autologo mentuaren ordezkapen kirurgia egitea da lehen aukerako tratamendua. Hala ere, prozedura horiek ez dira sarritan posible edota arrakastatsuak izaten eskuragarri dagoen ehun kopurua urria izan daitekeelako, edota beronekin lortutako morbilitatea handia izaten baita [8].

Alabaina, entesi edo tendoi/lotailu ehunak hezurretan txertatzen diren guneetan gertatutako patologien tratamendua are eta zailagoa izaten da. Interfase hauek ehun oso heterogeneo eta espezializatua osatzen dute, tendoi eta lotailuak hezurrarekin elkartzen diren eskualdeak izanik, giltzadurei egonkortasuna eta mugimendua ahalbidetzen diete. Entesien egituran bereizgarri dira hainbat osagaiek aurkezten dituzten gradienteak. Hala nola, ECMaren konposizioa, kolageno molekularen lerrokatzea eta osagai mineralak gradientez banatzen dira ehun batetik bestera [9]. Horrela, kolageno molekularen lerrokatzea tendoi/lotailu aldean nabariagoa den bezala, mineralen edukia handiagoa da hezur ehunean (2. Irudia).

Gainera, egiturazko ezaugarri horiek funtsezkoak dira entesiaren funtzioa egokia izan dadin: tendoi/lotailuen eta hezurren arteko tentsio mekanikoen transferentzia leuna bideratzea [10].



1. Irudia. Hezur hausturen ondorengo hezurraren birsortze prozesua. Hiru fasetan sailkatu daiteke hezurren sendaketa prozesua: (A) Hantura fasea, (B) Angiogenesiak bultzatutako kailu bigunaren eraketa, (C) Kailu gogorraren eraketa zelula amen desberdintzapen osteogenikoak bideratuta. Urrats bakoitza hainbat hakuntza faktorez, matrizeko seinale eta mota desberdinetako zelulez erregulatuta dago. Irudia [6] erreferentziatik baimenarekin moldatu da.



2. Irudia. Entesi fibrokartilaginosoen osaera eta egitura. Eskualde bakoitzeko matrizeko osagai eta zelula mota nagusiak adierazten dira. Kolageno zuntzen antolaketak eta mineralen edukiak egituran zehar alderantzizko joera erakusten dute. Irudia [9] erreferentziatik baimenarekin moldatu da.

Hala ere, eskualde konplexu espezializatu hauek eraberritze prozesuak sustatzeko gaitasun eskasa dute, bai kalte akutuen ostean edota endekapenezko gaixotasunen prozesu aurreratuetan. Kasu horietan, jatorrizko egituraren konposizio eta antolaketa ezberdina duen ehun fibrotikoa garatzen da, funtzio mekanikoak egoki betetzea galarazita egonik. Hori dela eta, hausturen errepikapen tasa handia izaten da eta epe luzerako ehunaren funtzionaltasuna eta emaitza klinikoak konprometituak egoten dira sarritan [11]. Gaur egun, lesio horien lehen aukerako tratamendu klinikoa mentuak kirurgikoki aplikatzean datza. Teknika kirurgikoetan hainbat aurrerapen lortu badira ere, interfazeen konponketen %95-ak huts egiten dutela kontsideratzen da, aurrez aipatutako ehun fibrotikoa eratzen delako [12].

Eskuragarri dauden ehunen urritasuna edota gaixoek garatutako errefusa, ordezko kirurgia mota guztiekin erlazionatzen diren arazoak dira. Hori dela eta, berebiziko ahaleginak egin izan dira erronka horiek gaindituko dituzten terapia berriak garatzeko, bereziki ehunen ingeniaritza izenarekin ezagutzen den alor berri baten aterpean eginiko esfortzuak nabarmentzen direlarik [13]. Kaltetutako ehunei irtenbide potentziala emateko helburua duen disziplina anitz biltzen dituen ikerketa alor moduan definitu daiteke. Zehazki, organo edo ehunen berezko funtzioa konpondu, ordezkatu, mantendu edota hobetzeko ordezko biologiko funtzionalen garapena sustatzera bideratzen da. Horretarako, seinaleztapen molekulak, zelulak eta biomaterialen konbinazioan oinarrituz artsenal terapeutiko zabala eskaintzen du. Medikuntzaren baitan, ehunen ingeniaritzak, ordezko tratamenduen bilakaeran aurrerapen

garrantzitsua suposatu du. Kaltetutako ehunak konpontzea ez ezik, organismoaren berezkoak diren birsortze mekanismoen estimulazioa lortu nahi da, zientziaren alor ezberdinetan eskuragarri dauden ezagutzei esker klinikan aplikagarria izan daitekeen erantzun bat emanez. Hori dela eta, kaltetutako ehunaren fisiologia eta fisiopatologiari buruzko ezagutza ezinbestekoa da primerako estrategiak garatu ahal izateko.

Ehunen ingeniaritzara bideratutako ordezko biologikoek bete beharreko eskakizunei dagokionez, zenbait ezaugarri orokorrak dira eta helburu ezberdinetara diseinatutako egiturek bete beharrekoak direla kontsideratzen da [14,15]. Horrenbestez, diseinaturiko ordezko oro biobateragarria izan beharko luke, ostalarian ehun konponketaren funtzioa erantzun immunologikorik eragin gabe betez. Gainera, materialak biodegradagarriak izatea komeni da, degradazio erritmoa ehun berriaren hazkuntzarekin bat etorriz. Horretaz gain, funtsezkoa da egiturek elkarren artean loturiko poroak izatea, zelulen barneratzea, elikagai eta hondakinen garraioa eta baskularizazio prozesuak baimendu ahal izateko. Ezaugarri orokor hauetaz gain, sortutako egiturek propietate bereziak eta espezifikoak izan beharko dituzte zein ehun konpontzera bideratuta dauden arabera. Horregatik, hezur akatsak [16] birsortzea edota tendoi-hezur interfazeen lesioak [17] tratatzea bada helburua, ezaugarri espezifikoak dituzten ordezkoak garatu behar dira. Hezur-ehunen ingeniaritzan bereziki garrantzitsua da biomaterial osteokonduktiboak garatzea. Bestalde, ehun-interfazeen ingeniaritzari dagokionez, entesi natiboaren fase anitzeko egitura antzeratzea da helburu nagusia. Gainera, mota ezberdineko agente terapeutikoen garraiatzaile gisa jarduteko bideragarritasuna lortzea biomaterialen diseinuan aintzat hartzeko ezaugarria da [18]. Agente terapeutiko gisa hazkuntza faktoreak ez ezik, zelulak ere kontsideratu daitezke.

Birsortze prozesuak aurrera eramango dituzten aldi baterako egiturak garatzeko, azken urteetan ikertu diren material guztien artean, biodegradagarriak diren jatorri naturaleko polimeroek interes berezia piztu dute. Material hauen abantaila nagusiak, gorputzean birxurgatuak izan baino lehen, ehunen hazkundea sustatu eta birmoldatzeko duten gaitasunak dira. Gelatina potentzial bioaktiboa duen etorkizun oparoko materiala da eta ostalariaren ehunarekin elkarrekintzak sortzeaz gain farmakoen eramaile gisa jarduteko sistemen diseinurako ere baliagarria da [19]. Alabaina, oraindik ere beharrezkoa da tenperatura fisiologikoan sare egonkorrak lortu eta materialaren berezko propietateak mantentzea ahalbidetzen duten erretikulazio estrategia eraginkorrak aurkitzea.

Ehunen ingeniaritzarako diseinatutako ordezkoek bete beharreko alderdi guztiak kontuan izanik, badirudi ezinezkoa dela material bakar batek baldintza guztiak betetzea. Horrenbestez, material desberdinak elkarrekin konbinatuz propietate sinergikoak dituzten konpositeak garatzea aztertu da alternatiba gisa [20]. Dena den, osagai kopuru minimoz osatutako konposite idealen diseinuak, erronka izaten jarraitzen du oraindik. Sinpletasun estrategia honek, fabrikazio eta sintesi konplexutasuna murrizteaz gain, araudi onarpena lortzea erraztu lezake.

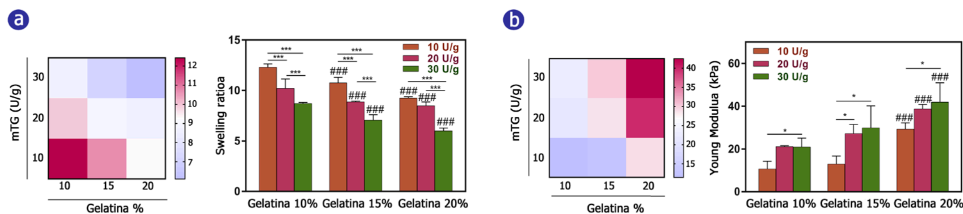
Ildo honi jarraituz, jatorri naturaleko polimeroak zeramika moduko beste material batzuekin konbinatu izan dira adibidez, polimero puruen propietate mekaniko ahulak eta hezurrekin integratzeko duten gaitasun mugatua gainditzeko helburuarekin.

Aspektu guzti hauek kontutan izanik, doktorego tesi hau gelatinak biomaterial moduan aritzeko duen gaitasunaren esplorazioan oinarritu da. Alde batetik, hezur-ehunen birsorkuntzarako hiru dimentsiotako (*three dimensional* edo 3D) sistema biobateragarrien garapena aztertu nahi izan da. Bestalde, tendoi eta hezur ehunen arteko interfazeen jatorrizko antolamendu konplexuak antzeratu ditzaketen hidrogel bifasikoen diseinua aurrera eramateko saiakerak egin dira.

Lehenengo urratsa, zelulen euskarri eta farmakoen garraiorako sistema gisa aritzeko 3D skaffold-ak lortu ahal izateko, gelatina kateen erretikulazioa gauzatzeko protokoloaren diseinua izan zen. Horretarako, *Streptoverticillium mobaraense* bakteriotik eratorritako transglutaminasa mikrobiarra (*microbial transglutaminase* edo mTG) entzima hautatu zen gelatinaren aminoazidoen arteko lotura kobalente egonkorra eragiteko. Entzima honek glutamato hondarren γ-karboxamida eta lisina hondarren ε-amino taldeen arteko azilo taldearen transferentzia erreakzioa katalizatzen du, aminoazido horien arteko lotura isopeptidikoa osatzen delarik. Ugaztun jatorriko transglutaminasekin alderatuz gero, ikerlan honetan erabilitako entzimak eskaintzen duen abantaila nagusiena elkargurutzaketa erreakzioa baldintza fisiologikoetan (37 °C, pH 7.4 eta ingurune urtsua) katalizatzeko gaitasuna da, hain zuzen ere kaltzio ioien beharrik gabe [21]. Hori dela eta, entzima hau erabiliz lortutako elkargurutzaketa prozesuarekin zelularik gabeko 3D sistemak garatzeaz gain, zelulak kapsularatuta dituen gelatinazko hidrogelak prestatzeko aukera ere badago [22].

Kasu honetan, tesi honen lehenengo atal esperimentalean zelula gabeko gelatinazko 3D skaffold-ak garatu ziren liofilizazio teknikan oinarrituz. Hain zuzen ere, gelatinaren hiru kontzentrazio (10, 15 eta 20 % (m/b)) eta hiru entzima proportzio (10, 20 eta 30 U/g gelatina) ezberdin erabiliz, bederatzi sistema ezberdin garatu ziren hasiera batean. Egitura horiekin burututako lehenengo ezaugarritze entseguen arabera, erabilitako gelatina eta mTG entzimaren proportzioek ura xurgatzeko gaitasunean eta konpresiozko ezaugarri mekanikoengan eragina dute.

Gelatina kontzentrazioa eta entzima aktibitatea zenbat eta altuagoa izan, ura hartzeko gaitasuna txikiagoa izan zen (3a. Irudia). Eragin horren atzean, polimero sare trinkoen eraketa eta ondoriozko uraren iragazkortasun eragozpena egon litezke [21]. Alderantziz baina, egituren zurruntasuna nabarmen handiagoa izan zen gelatina kontzentrazioa eta elkargurutzaketa maila handiagoak ziren kasutan (3b. Irudia). Horrenbestez, emaitza hauek gelatina kontzentrazioa eta mTG entzimaren jarduera katalitikoa egokituz 3D skaffold-en funtsezko ezaugarriak moldatzeko aukera dagoela utzi zuten agerian.



3. Irudia. Entzimatikoki erretikulatutako gelatinazko 3D skaffold-en ur hartze gaitasuna edo *swelling*-a (a) eta konpresio indarren menpeko propietate mekanikoen (b) karakterizazioa. Esanahia estatistikoa: * p<0.05, ** p<0.001 eta *** p<0.001 gelatina kontzentrazio berdineko taldeen artean eta # p<0.05, ## p<0.01 eta ### p<0.001 aktibitate entzimatiko berdineko taldeen artean, gelatina %10 duten egiturekin alderatuz.

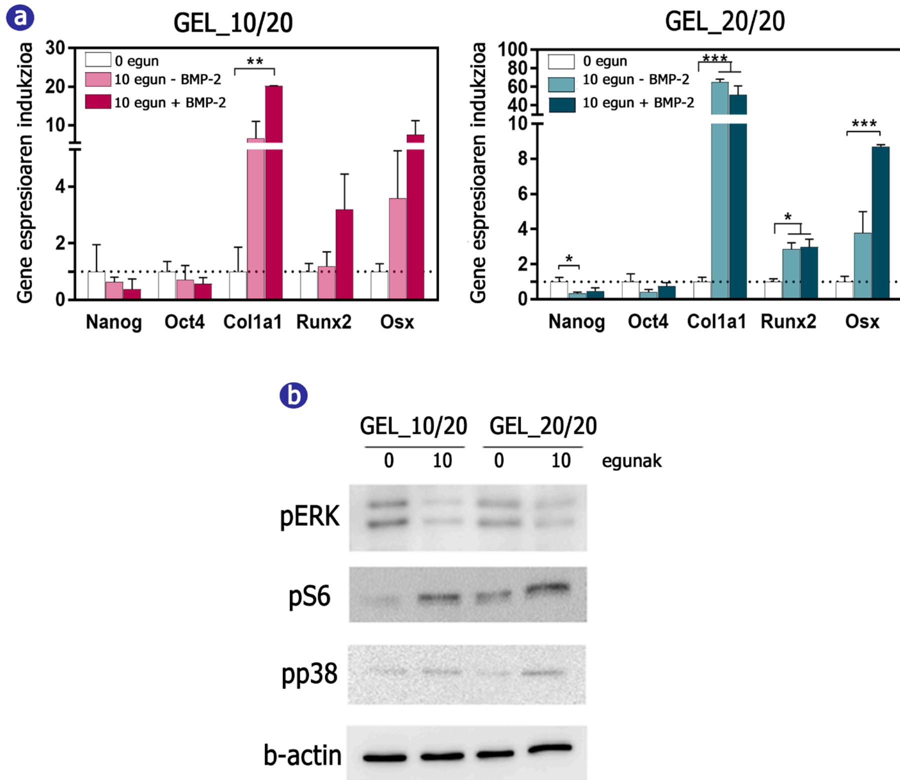
Jarraian, GEL_10/20 egiturak ura xurgatzeko gaitasuna eta GEL_20/20 sistemaren propietate mekanikoak kontuan izanik, bi formulazio hauek aukeratu ziren *in vitro* eginiko karakterizazioa osatzeko. Garatutako sistemen morfologia eta egitura mikroporotsua nolakoa zen ebaluatzeko asmoz, ekorketazko mikroskopio elektronikoa (*scanning electron microscopy* edo SEM) erabiliz laginen gainazala eta zeharkako guneen irudiak hartu ziren. Aztertutako bi laginen kasuan, esponja moduko egitura isotropoak lortu zirela baieztatu zen, gainazal osoan zehar sakabanaturik zituzten poroak antzeman baitziren. Hori dela eta, liofilizazio teknika gelatinazko 3D sistema porotsuak prestatzeko teknika egokia dela frogatu zen [23].

Gure hurrengo helburua, aukeratutako sistemen biobateragarritasun zelularra frogatu eta zelulen substratu gisa aritzeko gaitasuna ebaluatzea izan zen. Biobateragarritasuna gailu medikoen ebaluazio biologikoa egiteko ISO 100993 jarraibideak kontuan harturik aztertu zen eta garatutako skaffold guztiak biobateragarriak zirela ondorioztatu zen, lagin guztiek %70-eko bideragarritasun muga gainditu baitzuten zuzeneko eta zeharkako zitotoxikotasun azterketetan. Alabaina, lagin guztiekin zuzeneko zitotoxikotasun entseguan lortutako bideragarritasun balioak zeharkako azterketan lortutakoak baino txikiagoak izan zirenez, zelulak skaffold-etara atxikitu izana iradoki zen. Atxikimendu hori zelulen mintz plasmatikoko integrinen eta gelatinaren arginina-glizina-aspartatoa (a*rginine-glycine-aspartate* edo RGD) sekuentzien arteko elkarrekintzen ondorioz gerta litekela uste da.

Entzimatikoki erretikulatutako 3D gelatinazko egiturek zelulen atxikimendua eta proliferazioa baimentzeko gaitasuna sakonago ikertzeko asmoz, aurretik hezur-ehunen ingeniaritzako ikerlanetan erabili izandako hiru zelula-lerro ezberdin aukeratu ziren zelula eredu moduan [24,25]. Egun ezberdinetan burututako jarduera metabolikoaren neurketek eta nukleoen tindaketa entseguek agerian utzi zuten moduan, egituren gainazalean ereindako zelulek behar bezala proliferatu zuten. Hori dela eta, lan honetan garatutako sistemak zelulen euskarri izateko egokiak direla ondorioztatu zen.

Horretaz gain, skaffold horiek hezur birsorkuntzaren prozesuan izan dezaketen garrantzia ebaluatzeko asmoz, hezur-muineko zelula-ama mesenkimal murinoak (*murine bone marrow mesenchymal stem cells* edo mBM-MSCs) erabiliz, egiturek desberdintzapen osteogenikoa bultzatzeko duten gaitasunaren azterketa egin zen. Lortutako emaitzen arabera, zelulak 3D skaffold gainean erein eta 10 egunez kultibatu ondoren, zelula-amen bereizgarri diren geneen (*Oct4, Nanog)* espresio mailak murriztu egin ziren eta gene osteoblastikoen (*Col1a1, Runx2, Osx*) espresio maila modu esanguratsuan handitu. Zelulak hazteko erabilitako kultura medioari osagarri moduan hezur-proteina morfogenikoa -2 (*bone morphogenetic protein -2* edo BMP-2) hazkuntza faktore osteoinduktiboa gehitzean, aipatutako geneen espresio aldaketak are eta nabariagoak izan ziren (4a. Irudia). mBM-MSCs zelulen desberdintzapen prozesuan aktibatutako mekanismo zelularrak zeintzuk izan zitezkeen aztertzeko, 3D egituretan ereindako zeluletan aktibatutako seinaleztapen bideen gaineko ikerketa burutu genuen. Western-blot teknikan oinarrituz fosforilatutako ERK1/2 mailak txikiagotu eta fosforilatutako S6 kinasa eta p38 mailak handiagotu egin zirela ondorioztatu zen, *in vitro* hazkuntza 10 egunez mantendu ondoren. Emaitza hauek baliagarriak izan ziren zelula hauen proliferazio maila txikitu eta leinu osteoblastikora bideratutako joera piztu zela ondorioztatzeko (4b. Irudia) [26,27]. Izan ere, aurrez deskribatu izan den moduan BMP seinaleztapen ez-kanonikoa aktibatzen denean p38 proteinaren fosforilazioa gertatzen den bezala, S6 kinasaren fosforilazioa mTOR seinaleztapen bidearen baitan gertatutako prozesua dela ikusi da. Horrenbestez, molekula mailako prozesu hauek zelula-ama mesenkimalen desberdintzapen osteogenikoarekin erlazionatu izan dira [28,29].

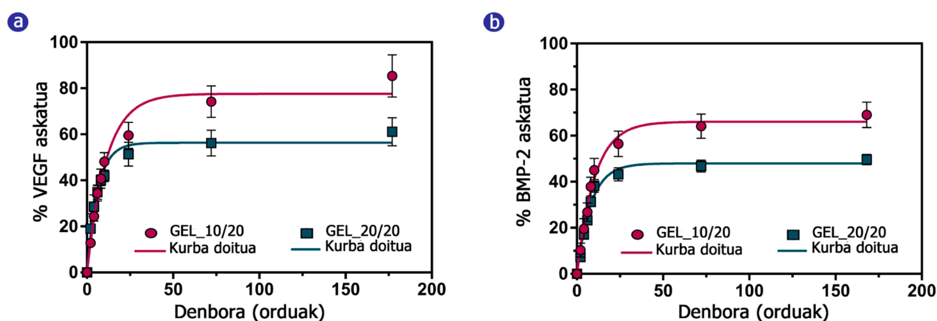
Amaitzeko, garatutako gelatinazko egiturek terapeutikoki aktiboak diren seinaleztapen molekulez kargatu eta euren ondorengo askapen iraunkorra bermatzeko duten gaitasuna aztertzeko, endotelio baskularraren hazkuntza faktorea (*vascular endotelial growth factor* edo VEGF) eta BMP-2 hazkuntza faktoreak erabili genituen. Aurrez prestatutako sistemetan faktore horiek adsortzioz kargatu ondoren, *in vitro* askapen entseguak burutu genituen. Bi hazkuntza faktore hauek hezur-ehunaren birmoldaketan duten eginkizuna dela, farmako eredu moduan aukeratu ziren [30, 31]. VEGF hazkuntza faktore angiogenikoa izanik, hezurren sendaketa prozesu biologikoan parte hartzen du, bere funtzio nagusia zelula endotelial sinusoidalen iragazkortasuna areagotzea da, birmoldaketaren hanturazko urratsean zelula neutrofiloen migrazioa induzituz [32]. Bestalde, aurrez aipatutako moduan, BMP-2 hazkuntza faktorea ezagutzen diren faktore osteoinduktibo potenteenetariko bat da, gaur egun klinikan hezur luzeen eta bizkarrezurreko fusio tratamenduetarako onartua egonik[33].



4. Irudia. 3D skaffold-ek hezur-muineko zelula-ama mesenkimal murinoetan (mBM-MSCs) markatzaile osteogenikoen espresioa handitzen dute. (a) BM-MSCs primarioak GEL_10/20 eta GEL_20/20 skaffold-en gainazalean erein eta hazkuntza medioa 2nM BMP-2-z osagarrituz 10 egunez mantendu ziren. *Nanog, Oct4, Col1a1, Runx2* eta *Osterix* geneen RNAm espresio mailak *Tbp* mailekin normalizatu ondoren aztertu ziren. (b) Fosforilatutako ERK, S6 kinasa eta p38 proteinaren adierazpen mailak Western-blot bidez antzemanda, b-aktina kargaren kontrol moduan erabiliz. Esanahia estatistikoa: * $p < 0.05$, ** $p < 0.01$ eta *** $p < 0.001$ 0 eguneko zelulekin alderatuz.

Bi faktoreen kasutan, kapsularatze efizientzia altuak lortu ziren, kargaturiko dosiaren ia %90 matrizeetan adsorbitzea lortu baitzen. Matrize hauen garapenerako erabilitako gelatinaren puntu isoelektrikoa 4.7-5.2 artekoa izanik, puntu isoelektriko altuagoko hazkuntza faktoreak (VEGF faktorearen puntu isoelektrikoa 8.5 da eta BMP-2arena 7.6) arrakastaz lotu eta askatzea lortu zela frogatu zen burututako entseguekin. Kasu guztietan, esperimentalki lortutako askapen perfilak lehen mailako kinetika eredura egoki doitu ahal izan ziren, molekula bioaktiboen askapena dosifikazio forman geratzen zen farmako kantitatearen proportzionala izanik [34]. 3D sistema hauekin hasierako bapateko askapena (*burst* efektua) ia %50-ekoa izan bazen ere lehenengo 24 orduetan, GEL_20/20 skaffold-ak hazkuntza faktoreak lotuta gehiago mantentzeko gaitasuna zuela frogatu zen. Izan ere, gelatina kantitatea handiagoa izanik kasu

horietan, karga negatibodun hazkuntza faktoreekin elkarrekintza elektrostatikoak gertatzeko aukera handiagoa da (5. Irudia) [35]. Bestalde, askapen entseguaren amaieran esperimentalki determinatutako askatutako ehuneko metatuak, erregresio ez-linealeko doikuntzan %VEGF $_{askatua\ \infty}$ eta %BMP-2 $_{askatua\ \infty}$ parametroentzat estimatutako balioekin bat etorri ziren. Emaitza horien arabera, difusioz gertatutako faktoreen askapen osoa lortu zen entseguaren amaieran. Horretaz gain, benetako baldintza fisiologikoetan skaffold-en degradazioa tarteko, askatu gabe geratu ziren hazkuntza faktoreen ematea gertatu daitekeela aurreikusten da [36].



5. Irudia. Entzimatikoki erretikulatutako 3D gelatina skaffold-etatik bideratutako endotelio baskularraren hazkuntza faktorea (*vascular endothelial growth factor* edo VEGF) (a) eta hezur-proteina morfogenikoa-2 (*bone morphogenetic protein -2* edo BMP-2) (b) *in vitro* askapena. Askapen metatuaren emaitza esperimentalak eta lehen mailako askapen kinetikaren doikuntza kurba hazkuntza faktore bakoitzarentzat irudikatuta.

Aurkikuntza hauek guztiak kontutan harturik, hezur-ehunen ingeniaritzarako gelatinan oinarritutako biomaterial konpositeak diseinatzerako potentzial handiena izan zezakeen hautagaia GEL_20/20 skaffold-a zela kontsideratu genuen. Nahiz eta formulazio honek zelulen atxikimendua eta farmakoen askapena baimentzeko ezaugarriak zituela frogatu, bereziki garrantzitsua da 3D skaffold-en berezko ezaugarri osteokonduktiboak indartzea. Izan ere, hezurren birmoldaketa bultzatzeko berezko gaitasuna duten sistemekin erabili beharreko BMP-2 dosiak txikiagoak izan litezke. Horrela, hazkuntza faktore hau fisiologikoak diren dosien gainetik erabiltzeak dakarren eragin desiragaitzen agerpena saihestea lortu liteke.

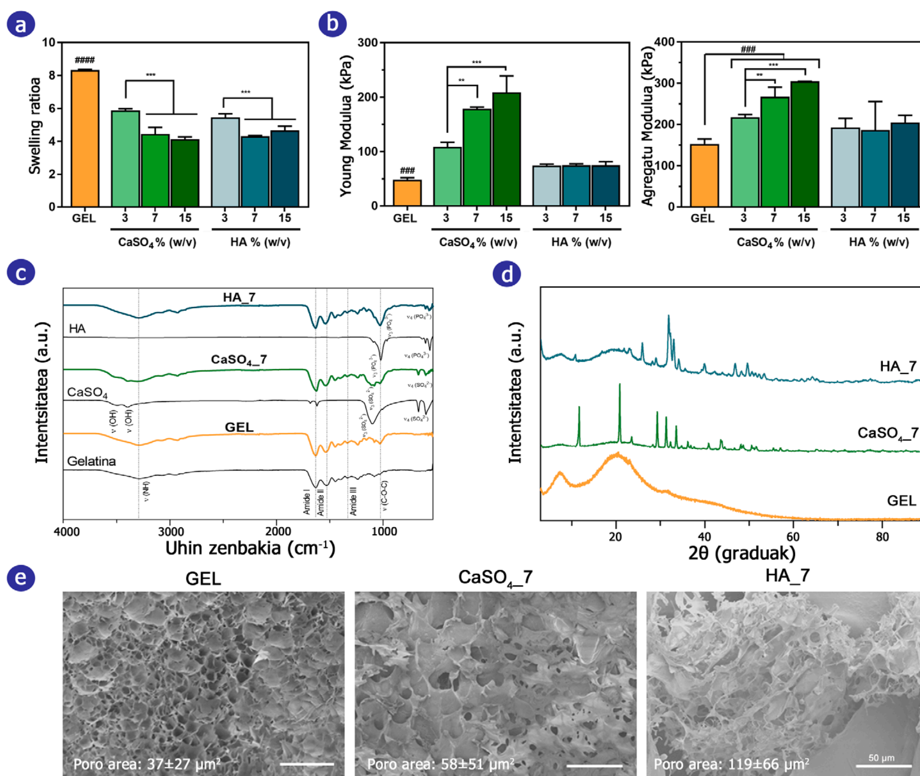
Doktorego tesi honen bigarren urratsean, hezur-ehunaren ingeniaritzara bideratutako gelatinan oinarritutako biomaterial konpositeen garapena eta karakterizazioa izan ziren helburu nagusiak. Gainera, hezurrak konpontzeko baldintza bereziki zailak eragiten dituen gaixotasunean jarri genuen arreta, osteoporosian hain zuzen ere. Helburu hori aurrera eramateko, hezur-ehun natiboaren konposaketa hobeto mimetizatuko zuten konposite organiko/inorganikoz osatutako 3D skaffold-ak garatu ziren. Osagai organiko moduan, aipatutako lehen ikerketa lanean optimizatutako formulazioa (GEL_20/20) erabili genuen. Aldiz, osagai inorganikoari dagokionez, hidroxiapatita (*hydroxyapatite* edo HA) eta kaltzio sulfato

biozeramikak erabiltzea aukeratu zen. Azken material hauek aurrez hezur betegarri moduan erabili badira ere, material bakun moduan ehunen ingeniaritzarako ordezko biologikoak garatzeko hainbat muga aurkezten dituzte. Besteak beste, HA birxurgatzeko arazoak, material hauskorrak izanik finkapen kirurgiko egonkorrak eragiteko zailtasunak edota mentuen erdiguneko zatian baskularizazioa sortzeko ezintasuna aipatu daitezke material hauen desabantailen artean[37].

Lehendabizi, material biozeramikoak modu graduatuan gelatina sareetan murgilduz sistema konposatuak sortu genituen. Hiru sendotasun maila ezberdineko egiturak garatu ziren, material betegarrien kontzentrazio mailakatuak erabiliz. Sendotasuna lortzeko erabilitako fase inorganikoaren presentziak, garatutako skaffold-en ur hartze gaitasunean, degradagarritasunean eta propietate mekanikoetan zuen eragina aztertu zen. Garatutako prototipo guztiek ur hartzeko gaitasun egokia izatea erakutsi bazuten ere, *swelling* ratioa sendotasun mailari proportzionalki txikiagotu zen (6a. Irudia). Bestalde, hidrosolugarritasun desberdineko gatz inorganikoak erabiltzeak, egituren *in vitro* degradazio hidrolitikoan eragina izan zuen. Sistema guztiek degradazio hidrolitikoaren profil bifasikoa aurkeztu zuten, seguruenik higadura fisiko eta oligomeroen difusio eta disoluzio prozesuen ondorioz. Hala ere, apatita zeramikaz sendotutako skaffold-ek uretan egonkortasun handiagoa izatea erakutsi zuten, kaltzio sulfatoarekin sendotutako egiturekin alderatuz [38]. Gainera, skaffold guztiak kolagenasa entzimadun disoluzioan murgiltzean guztiz degradatu zirenez, egitura horiek *in vivo* inplantatu ondoren ECM birmoldatzen duten proteasen ondorioz degradatuak izango direla aurreikusi zen [39]. Degradazio horren ondorioz, konposite skaffold-etatik kaltzio ioien askapena gertatu liteke, ingurunean efektu parakrinoa sortuz. Hori dela eta, osteoblastoetan aintzindariak diren zelula endogenoak lesioaren gunean biltzea lortzen bada, desberdintzapen osteogenikoa bultzatzeko aukera areagotuko litzateke [40].

Propietate mekanikoei dagokionez, ardatz bakarreko konpresio estatiko entseguak burutu ziren egitura bakoitzaren Young modulua eta Agregatu modulua zehazteko asmoz (6b. Irudia). Kasu guztietan entsegu konfinatuetan lorturiko Agregatu modulua, Young modulua baino handiagoa izan zen. Emaitza hauekin, skaffold-etan konpresio ez-konfinatua eragitean alboranzko hedapena gertatzen dela ondorioztatu daiteke [41]. Aukeratutako bi biozeramika motak gehitzearekin gelatina skaffold-en sendotze efektua lortu bazen ere, HArekin ez bezala, kaltzio sulfatoaren kasuan efektu hori gehitutako gatz kontzentrazioarekiko proportzionala izan zen. Aipatzekoa da, hezur-ehunen ingeniaritzako 3D skaffold polimerikoek izan beharreko zurruntasun optimoari buruzko adostasun orokorturik egon ez arren, hainbat dira 60 kPa inguruko Young modulua duren hidrogelek MSCen prozesu osteogenikoa *in vitro* eta *in vivo* bultzatu dezaketela frogatu duten ikerketak. Alabaina, Young moduluaren balore altuagoak (126-181 kPa) prozedura kirurgiko hobea eta ordezkoaren finkapen egonkorrak lortzearekin erlazionatu dira [42, 43].

Aurkikuntza horiek guztiek, entzimatikoki erretikulatutako gelatina sarean kaltziodun biozeramikak gehituaz garatutako sistemen propietateak erraz egokitu daitezkeela iradokitzen dute. Hori dela eta, gelatinan oinarritutako konposite skaffold-ek funtsezko ezaugarri egokiak biltzen zituztela ondorioztatu genuenez, potentzial terapeutikoaren karakterizazioan eta ebaluazioan sakontzen jarraitu genuen. Hala ere, *in vitro* karakterizazio osagarria, jokabide biologikoaren ebaluazioa eta hezurren birsorkuntza gaitasunaren azterketa, kaltzio gatzak %7 (m/b) kontzentrazioan zituzten skaffold konpositeekin soilik burutu genuen. Kontrol moduan, soilik gelatinaz osatutako egiturak aintzat hartu genituen.



6. Irudia. Gelatina/biozeramika konpositez prestatutako skaffold-en karakterizazio fisiko-kimikoa. (a) *Swelling* ratioa eta (b) konpresiozko propietate mekanikoen zehaztapena gradualki sendotutako sistemetan. Garatutako konposite skaffold-en FTIR espektroak (c) eta XRD patroiak (d). Liofilizatutako skaffold-en SEM irudi adierazgarriak. HA: hidroxiapatita. Esanahia estatistikoa: ** $p<0.01$ eta *** $p<0.001$; ### $p<0.001$ eta #### $p<0.0001$ GEL eta sendotutako gainontzeko taldeen artean.

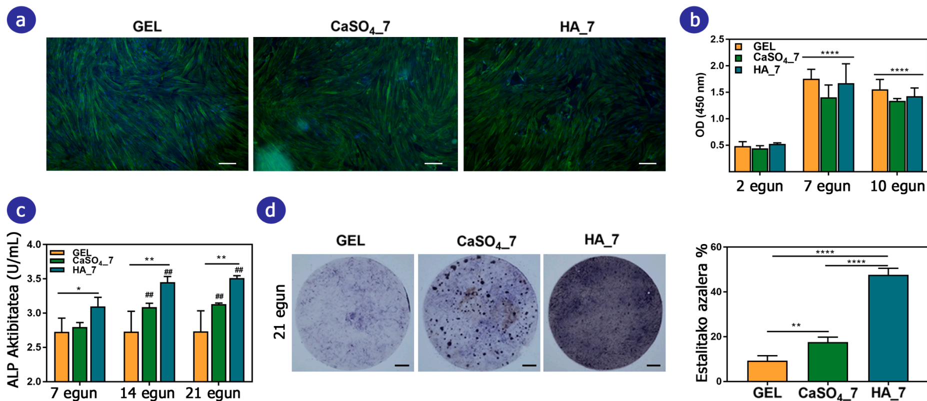
Aukeratutako sistemen mikroegitura eta karakterizazio kimikoa, SEM irudiekin, X izpien dispertsioaren energia espektroskopia (*energy dispersive X-ray spectroscopy* edo EDAX), Fourieren transformatu bidezko espektroskopio infragorria (f*ourier transform infrared spectroscopy* edo FTIR) eta X izpien difrakzioaren (*X-ray diffraction* edo XRD) analisiaren bidez osatu ziren. Liofilizatutako laginen gainean EDAX mapatzeak agerian utzi zuen kaltzio elementuaren presentzia sendotutako skaffold guztietan eta sufre eta fosforo elementuak kaltzio sulfatoarekin eta HArekin sendotutako sistemetan, hurrenez hurren. Horretaz gain, HAri eta kaltzio sulfatoari FTIR eta XRD espektroetan (6c,d. Irudia) atxikitzen zaizkien pikoak, garatutako gelatinazko konposite skaffold-etan antzeman ziren, egituretan sendotzaile mineralen presentzia baieztatuz. Bestalde, SEM irudiek erakutsi zuten garatutako egiturak porotsuak zirela eta aukeratutako material sendotzaile biak gehituz poro tamaina handitzea lortu zela, HAren kasuan efektua nabariagoa izanik (6e. Irudia). Gelatina/HA konposite skaffold-en kasuan 100 µm tamainako poroak antzeman ziren eta poro tamaina honetako egiturek osteoblastoen migrazioa eta proliferazioa bultzatu dezaketela frogatu izan da [44]. Horrenbestez, ikerketa lan honetan garatutako sistemek hezur-ehuna konpontzeko prozesua bultzatzeko ezaugarri interesgarriak biltzen dituztela ondorioztatu zen.

Jarraian, garatutako skaffold-ek *in vivo* hezur ehunaren konponketa bideratzeko gaitasuna aztertu aurretik, giza hezur-muineko zelula-ama mesenkimalak (*human bone-marrow derived mesenchymal stem cells* edo hBM-MSC) erabiliz, sistemen errendimendu biologikoaren azterketa burutu zen *in vitro*. Zelula primario hauek oso erabiliak izan dira 3D skaffold-en ezaugarri osteoinduktiboak aztertzeko, besteak beste eskuragarritasun egokia, desberdintzapen osteogeniko handia eta erreplikazio potentzial nabaria eskaintzen dutelako [45]. Hasteko, aurrez aipatutako ISO 10993 jarraibideak kontuan izanik, biobateragarritasunaren aurretiazko ebaluazioa burutu zen. Kaltzio fosfatoz osatutako biomaterial batzuekin hainbat efektu toxiko antzeman izan badira ere hazkuntza medioaren konposaketa ionikoan eraginiko aldaketen ondorioz [46], ikerlan honetan gelatina konpositez garatutako sistema guztien bideragarritasun balioek biomaterial ez-zitotoxikoentzat ezarritako muga gainditu zuten.

Ondoren, garatutako skaffold-ek hBM-MSC zelulen atxikimendua, proliferazioa eta desberdintzapen osteogenikoa sustatzeko gaitasuna ebaluatzeko, zelulak aurrez prestatutako skaffold hidratatuen gainazalean erein ziren eta hazkuntza medio basala erabiliz kultibatu. Zelulen bideragarritasuna aztertzeko asmoz, ereintza ondorengo 2 eta 10 egunetan zelulak bizirik ala hilda dauden determinatzea baimentzen duen tindaketa burutu zen. Lortutako immunofluoreszentzi irudiek eta fluxuzko zitometriaren analisiek erakutsi zuten, zelulek bideragarritasun tasa altuak mantentzen zituztela garatutako sistemetan, talde esperimentalen artean ezberdintasun nabarmenik gabe.

Zelulen nukleoa eta zitoeskeletoa fluoreszentzia bidez tindatu ostean, zelulak skaffold-en gainean erabat atxikituta eta zabalduta zeudela antzeman genuen (7a. Irudia). Emaitza hauen

arabera, biozeramika betegarriak gelatina sarean gehitzeak ez du eraginik zelulen mintzetako integrina errezeptore eta gelatinaren RGD sekuentzien arteko elkarrekintzetan. Gainera, jarduera metabolikoen analisiek aditzera eman zutenez, ereindako zelulak proliferatzeko gaitasuna erakutsi zuten batez ere lehenengo astean, prestatutako konposite skaffold-en artean ezberdintasunik antzeman gabe (7b. Irudia). Horregatik, ikerketa lan honetan aktibitate zelularraren euskarri izan daitezkeen plataformak arrakastaz diseinatu zirela ondorioztatu zen. Zelula hauek ainguraketa menpekoak izanik, beraien bideragarritasuna eta potentzial osteogenikoa mantentzeko gainazaletan atxikita egon behar dute. Hori dela eta, prestatutako egituretan lortutako atxikipen egokia tarteko, hBM-MSCak skaffold gainean erein ondoren bereizketa osteogenikorako gaitasuna mantendu edota sustatu egin litekeelaren hipotesia osatu genuen [40].



7. Irudia. Garatutako skaffold gainean ereindako giza hezur-muineko zelula-ama mesenkimalen (hBM-MSC) atxikipen, proliferazio eta konpromiso osteogenikoaren azterketa. (a) hBM-MSC zelulen nukleoa (urdinez) eta F-aktinaren (berdez) fluoreszentzia irudiak, 7 egunez hazi ondoren (eskala = 200 µm). (b) Skaffold-etan ereindako zelulen jarduera metabolikoa 10 egunez. (c) Jariatutako fosfatasa alkalinoaren (*alkaline phosphatase* edo ALP) aktibitatea 7, 14 eta 21 egunez kultibatu ondoren. (d) ALP tindaketaren irudiak eta kuantifikazio erlatiboa zelula kultiboa 21 egunez mantendu ondoren (eskala = 1.5 mm). Esanahia estatistikoa: * p<0.05, ** p<0.01, *** p<0.001 eta **** p<0.0001; ## p<0.01 skaffold berdinaren balioa 7. egunean alderatuta.

Hipotesi hura frogatzeko, gure hurrengo helburua zelula-amen eta konpromiso osteogenikoarekin erlazionatutako adierazleen espresio mailan oinarrituz 3D skaffold-etan ereindako hBM-MSCen desberdintzapen prozesua ebaluatzea izan zen. Alde batetik, jariatutako fosfatasa alkalinoaren (*alkaline phosphatase* edo ALP) aktibitatea neurtu zen eta bestalde, 5 gene ezberdinen espresio mailak determinatu ziren [47, 48].

Zelula-amen adierazgarri diren geneen (*Nanog, Oct4*) espresio mailak egitura guztietan denborarekin murriztu egin ziren bitartean, konposite organiko/inorganikoetan ereindako zelulen ALP jarduera areagotu egin zen (7c,d. Irudia). Alabaina, emaitza kontrajarriak lortu ziren gene osteogenikoen espresio mailen azterketan. Alde batetik, *Col1a1* eta *Fmod* adierazle osteogenikoen gene espresio mailak areagotu egin ziren arren, batez ere gelatina/HA skaffold-en kasuan, *Runx2* transkripzio faktorearen adierazpen maila egonkor mantendu zen talde esperimental guztietan. Hain zuzen ere, transkripzio faktore hau MSC zelulen desberdintzapen osteogenikoaren funtsezko erregulatzailea kontsideratzen da. Transkripzio faktore hau hainbat bide molekular desberdinetan inplikatuta dagoela deskribatu da, hala nola BMP kanoniko eta ez-kanonikoan, Wnt/β-katenina edota ERK1/2 seinaleztapen bideetan [28,48]. Hala ere, desberdintzapen osteogenikoa bideratzeko bestelako mekanismo molekularren parte hartzea ikertu eta deskribatzeke egon litezke [49,50]. Adibidez, orain gutxi eginiko ikerketa batzuek ondorioztatu duten moduan, *Osterix* transkripzio faktorearen eta NFATc1 kofaktorearen arteko elkarrekintza zuzenak *Col1a1* genearen promotorearen aktibazioa sustatu dezakete, *Runx2*-aren menpeko transkripzioa aktibatu gabe [51,52]. Hori dela eta, gure datuen arabera, ikerketa lan honetan garatutako 3D konposite skaffold-ek hBM-MSCen bereizketa prozesuan eragina izan dezakete, leinu osteogenikoranzko konpromisoa bultzatuaz hain zuzen ere. Hala ere, komenigarria izango litzateke ikerketa gehiago eginez prozesu horretan martxan jarritako mekanismoak ezagutzea. Kasu horretan, zelulen eta biomaterial arteko elkarrekintzen gaineko jakintza sakonagoa lortuko litzateke.

Ildo horretatik jarraituz, doktorego tesiaren hurrengo urratsa, zelula eta skaffold-en artean gertatutako elkarrekintzen gertakari molekularrak ikertzeko asmoz, azterketa proteomikoaren potentziala baloratzea izan zen. Soilik proteina kopuru mugatuen ekintza ikertzeko egokiak diren beste teknika batzuk ez bezala, tandem masa espektrometriara loturiko kromatografia likidoa eskala handiko proteinen karakterizazioa burutzeko tresna egokia dela kontsideratzen da, proteoma osoaren ebaluazio globala eta integratua egiteko aukera eskaintzen duelarik [53]. Dena den, gisa honetako ikerketetan datu eta ondorio baliotsuak lortu ahal izateko, ezinbestekoa da laginen prozesamendu eta datuen azterketarako metodologiazko baldintzen optimizazioa burutzea, erabilitako zelula eta biomaterial espezifiko bakoitzean arreta jarriz. Kasu honetan, guk garatutako 3D skaffold-ak izaera proteikoa zutela kontuan harturik, azterketa proteomiko egokia burutzeko baldintzak ezartzea bereziki zaila izan liteke. Hori dela eta, zelulen profil proteomikoa lortu ahal izateko beharrezko baldintza zehatzak finkatzeko asmoz, *in vitro* zelulen kultibo baldintzak, proteinen eluzio protokoloak eta bi masa espektrometria teknologia ezberdin aztertu genituen.

Zelulen proteinen eluzioa lortzeko abiapuntu egokia PBSarekin eginiko skaffold-en garbiketak izan daitezkeela ikusi genuen. Modu horretan, hazkuntza medioak dituen serum proteinak baztertzea lortu daiteke neurri batean. Ondoren, zelulen lisia 7M urea, 2M tiourea eta 4% 3-[(3-kolamidopropil) dimetilamonio]-1-propansulfonatoa (CHAPS) erabiliz

prestatutako tanpoiarekin eragin zen, digestio triptikoa egin baino lehen. Protokolo hau jarraituz garatutako skaffold-etatik behi jatorriko proteinen eluzioa ere lortu zen arren, azken belaunaldiko timsTOF Pro masa espektrometroa erabiliz lagin bakoitzean oso proteina kopuru altua identifikatzea lortu genuen. Espektrometro honen sentikortasun eta bereizmen bikainaren laguntzaz, skaffold bakoitzetik eluitutako milaka proteina identifikatzea lortu zen. Gainera, serumik ez duen hMSC zelulen espantsiotarako bereziki formulatutako hazkuntza medio komertzialarekin hainbat proba egin baziren ere, ez zen lortu behi jatorriko proteinen proportzioa murriztea. Hala ere, giza jatorriko eta behi jatorriko proteinen proportzioak berdin mantendu zirenez talde esperimental guztietan (identifikatutako proteina guztien %80 giza jatorrikoak zirela baieztatu zen), laginen arteko analisi kuantitatiboan aurrerapausoak emateko aukera izan genuen.

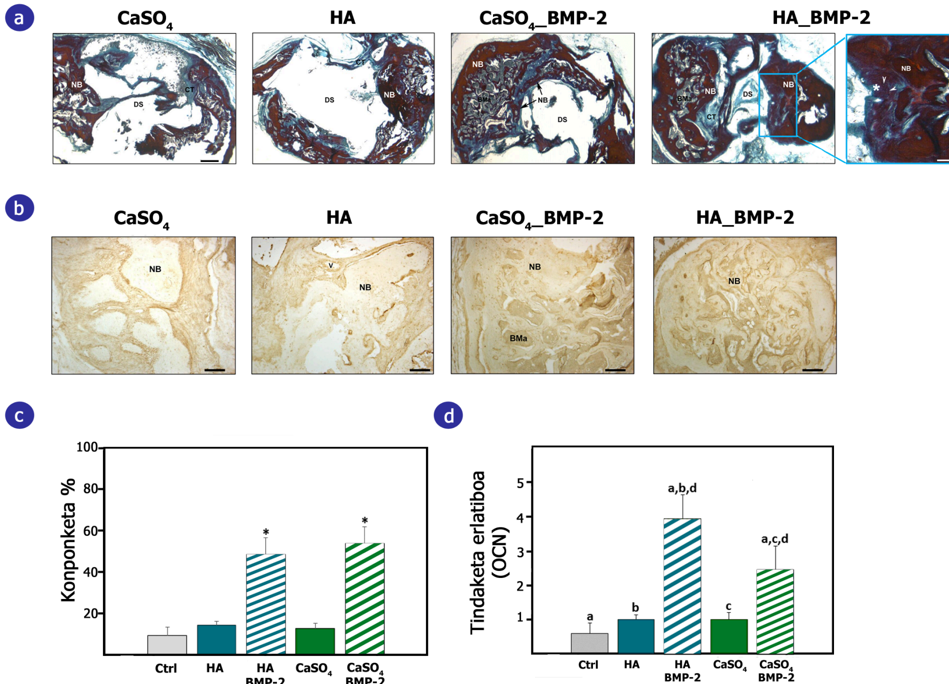
Proteinen identifikazioa egiteko PEAKS® softwarea erabili genuen, eta PEAKSQ modulua azterketa kuantitatiboentzat. Gutxienez jatorriaren araberako bi peptido esklusiborekin identifikatutako proteinak hartu ziren kontutan analisiak egiteko eta proteina bakoitzak lagin bakoitzean aurkeztutako intentsitateak kalkulatu ziren. Skaffold desberdinetan ereindako zelulen profil proteomikoak konparatzeko asmoz, Perseus softwarea erabiliz lagin eta proteina bakoitzari esleitutako intentsitateen arteko analisia egin zen. Horrenbestez, ugaritasunaren antzekotasunean oinarrituz proteinak eta laginak taldekatu egin ziren eta lortutako multzoen arabera, zelulak hazteko erabilitako skaffold-en konposizioak hBM-MSC zelulen profil proteomikoan eragina izan dezakela ondorioztatu genuen. Puntu honetara iritsita, interesgarria gerta dakiguke *Gene Ontology* eta *Ingenuity Pathway Analysis* moduko baliabide informatikoak erabiliz talde esperimental bakoitzeko proteina diferentzialak inplikatuta dauden funtzio biologikoen inguruko ezagutzan sakontzea. Beraz, masa espektrometria bidezko azterketa proteomikoa burutzeko baldintzak arrakastaz finkatzea lortu zen. Lagin kopurua handituz eta pertsona desberdinen zelulak erabiliz eginiko entseguekin, garatutako konposite skaffold-etan ereindako hBM-MSC zelulei buruzko informazio erabakigarria lortu daitekeela aurreikusten dugu.

Gelatina konpositeen biobateragarritasuna aztertu eta gainazalean ereindako hBM-MSC zelulen aktibitatea ikertu ondoren, gure hurrengo urratsa zelularik ez zuten plataforma osteokonduktiboak faktore terapeutiko osteoinduktiboarekin funtzionalizatzea izan zen, farmakoen askapenerako funtzioa bete zezaten. Lehendabizi 3D skaffold-ak BMP-2 hazkuntza faktorearekin kargatu eta beraien *in vitro* askapen profila nolakoa zen aztertu genuen. Kaltzio sulfato eta HA biozeramikak formulazioetan gehitzeak ez zuen gelatinaren hazkuntza faktorea adsorbatzeko gaitasunean eraginik izan. Hasieran antzemandako bapateko askapena (*burst* askapena), kargaturiko dosiaren %35-50 bitartekoa izan zen lehen bi egunetan. Emaitza horien arabera, biozeramiken presentziak bapateko askapenaren denboran zeharreko luzapen txikia eragin dezake. Efektu horren arrazoia, sulfato eta fosfato talde anioniko eta BMP-2 faktorearen talde kationikoen artean gertatutako elkarrekintza elektrostatiko ez-espezifikoak izan

daitezkeela uste dugu [54]. Bapateko askapen horren ondoren, hazkuntza faktorearen askapena sendotutako gelatinazko bi skaffold-etatik mantendua izan zen eta 2 asteren buruan kargaturiko dosiaren %60 askatu zela determinatu zen. Hortaz, ikerlan honetan garatutako sendotutako gelatinazko 3D skaffold-ak faktore osteoinduktibo honen garraiorako egokiak izan litezkeela ondorioztatu genuen, lesioaren lekuan agente terapeutikoaren espazio-denborazko kontrola handitzea lorturik administratu beharreko dosia murriztuz. Hori dela eta, proposaturiko ikuspegi hau, fisiologikoak baino altuagoak diren dosiekin erlazionaturiko eragin desiragaitzak saihesteko irtenbidea izan liteke.

Hipotesi hori frogatzeko asmoz, gure hurrengo helburua BMP-2 dosi baxuekin kargaturiko gelatina konpositez osatutako skaffold-ek sagu osteoporotikoen garezur-gangan tamaina kritikoko akatsak birsortzeko gaitasuna aztertzea izan zen. Hezur-ehunen ingeniaritzaren baitan garatutako ordezkoen gaitasun terapeutikoa frogatzeko garaian aukeratutako animalia ereduek berebiziko garrantzia dute. Izan ere, hezur birsorkuntzari buruzko emaitza oso ezberdinak lortu izan dira BMP-2arekin kargaturiko formulazio berdina erabiliz arratoi osasuntsu eta osteoporotikoekin eginiko ikerketetan. Hori dela eta, gure estrategia erronka berezia suposatzen duen animalia eredua erabiltzea izan zen, ikerlan honetan garatutako gelatinazko egituren potentziala aztertzeko. Osteoporosiarekin erlazionaturiko hezur ehunaren konponketarako atzerapena, tamaina kritikoko hezur akatsarekin konbinatuz gero, berezko hezur birsorkuntza mugatua dagoen egoera osatzen da. *In vivo* ikerketarako erabilitako osteoporosi indukzio eredua aurrez balidatua izan da, garezur-gangako hezurraren egiturazko ezaugarriak aldatu egiten direla frogatuz [55]. Skaffold-ak 600 ng BMP-2arekin kargatu eta sagu osteoporotikoen garezur-gangan eginiko 4 mm-ko diametroko akatsean ezarri ziren.

3D skaffold-en eragin terapeutikoa aztertzeko helburuarekin, akatsen eremua histologia eta histomorfometria bidez ezabaluatu zen inplantazioa egin eta 8 asteetara. Hezur-ehun berria eta hezurraren mikroegitura talde esperimental guztietan nabaritu baziren ere (8a,c. Irudia), skaffold-ak hutsik inplantatu ziren kasuetan ehunaren birsorkuntza akatsen ertzetan soilik antzeman zen. Aitzitik, BMP-2 hazkuntza faktorearekin kargaturiko 3D skaffold-ak administratu ziren kasutan hezurraren birsorkuntza prozesua nabarmenki areagotu zen eta ia akatsaren azalera erdia hezur ehun berriarekin estali zen. Gelatina/HA skaffold funtzionalizatuen kasuan, egitura trinkoagoa eta ECMaren eskualde mineralizatuak antzemateaz gain, osteozitoen antzeko zelulak ikusi ziren birsortutako ehun berriaren baitan. Gainera, hazkuntza faktoredun skaffold-ak inplantatu ziren kasutan, osteokaltzinaren (*osteocalcin* edo OCN) espresio mailak handiagoak izan ziren (8b,d. Irudia). OCN, hain zuzen ere, mineralizazioaren eta osteogenesi prozesuaren adierazle berantiarra izanagatik ezagutzen da eta HA_BMP-2 formulazioak $CaSO_4$_BMP-2 kasuan baino seinale nabariagoa lortu zuen.



8. Irudia. Hezur birsorkuntzaren ebaluazioa sagu osteoporotikoen garezur-gangako akatsetan, garatutako 3D skaffold-ak ezarri eta 8 asteetara. (a) Aztertutako talde esperimental guztien VOF tindaketaren irudi adierazgarriak. HA_BMP-2 taldean urdinez nabarmendutako eskualdean, matrize estrazelularraren mineralizazio goiztiarra antzematen da gorriz tindatutako gunean (* irudikatua), osteozitoen antzeko zelulak hautematen direlarik (geziek irudikatua). Irudi panoramikoen eskala = 500 µm. Xehetasun handiko irudiaren eskala = 50 µm. (b) Akats eremuan osteokaltzina (*osteocalcin* edo OCN) tindaketa espezifikoaren irudi adierazgarriak. Eskala = 100 µm. (c) Talde bakoitzean lortutako konponketa portzentaia histomorfometria azterketa kuantitatiboaren arabera. Esanahia estatistikoa: * p<0.001 kontrol taldearekin alderatuta. (d) Talde esperimental bakoitzean OCN tindaketa erlatiboaren kuantifikazioa, unitate arbitrarioetan erakutsia. Letra bereko taldeen artean desberdintasun estatistikoa antzeman da (p<0.001). BMa: Hezur-muina, CT: Ehun konektiboa, DS: Akats eremua, NB: sortutako ehun berria, v: odol hodia

Bigarren ikerlan esperimentalaren emaitza guztiak orokorrean harturik, farmako osteoinduktiboen eramaile moduan aritzeko biozeramikak erabiliz egokitutako gelatinazko 3D sistemen gaitasuna frogatu genuen. Gainera, hezur birsorkuntzaren egiaztapena bereziki zailtzen duen gaixotutako ingurunean burutu zen, tamaina kritikoko hezur osteoporotikoen akatsetan hain zuzen ere.

Gelatina/HA konposite biomimetikoarekin lortutako emaitza itxaropentsuak aintzat harturik, doktorego tesiaren hurrengo urratsean, mineralizatutako gelatina konpositeak eta egitura anisotropodun hidrogel bioinspiratuak sistema bakarrean integratzea izan genuen helburu. Hain zuzen ere, seinale biokimikoak eta egiturazko ezaugarri bereziak espazioan bereiztuak dituen egituren garapena izan genuen jomugan. Proposaturiko estrategia hori interfaze-ehunen ingeniaritzaren ikuspegi moduan sailkatu daiteke. Izan ere, mota horretako ikerlanen helburu nagusia interfaze muskuloeskeletikoetan, hezur-tendoi arteko entesietan esaterako, berez aurkezten diren konposizio, arkitektura eta zelulen antolaketa bereiziak imitatzen dituzten egituren garapena da. *Ex vivo* prestaturiko egituren osagai espezifikoak modulatzeko funtzionaltasunak gehitzeak, ehun-interfazeetarako diseinatutako biomaterialen propietateak egokitzea ahalbidetu dezake [56].

Balizko suposizio horretatik abiatuta, tesiaren hurrengo saiakeran, interfazeko fase bakoitza simulatuko zituzten konposatuak neurrira ekoizteko asmoz, gelatina erabili genuen biomaterial nagusi gisa eta tamaina nano eta mikrometrikoko betegarri espezifikoekin konbinatu genuen. Horrenbestez, gelatinan oinarritutako hidrogelen konposizioa eta fabrikatzeko baldintzen egokitzea izan ziren ikerlan horretan jarraitutako estrategia nagusiak.

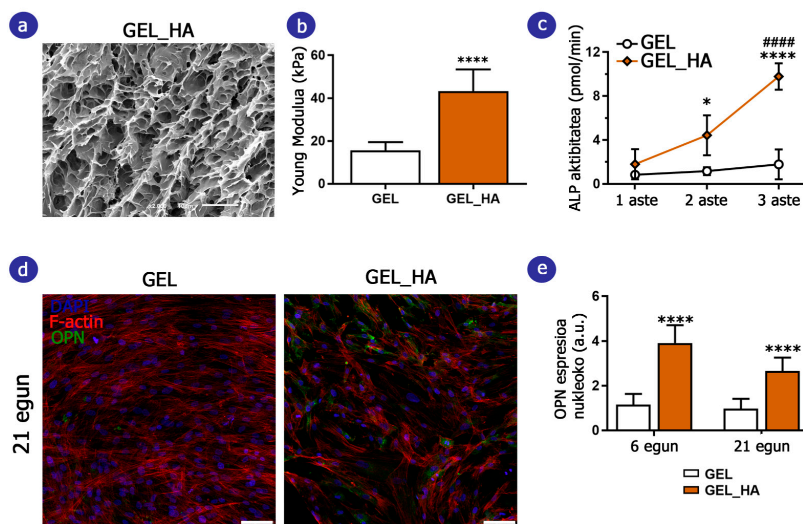
Tesi honetan aurretik egindako lanen moduan, kasu honetan ere gelatinaren erretikulazioa mTG entzima erabiliz osatu zen. Gainera, erretikulazio entzimatikoak baldintza fisiologikoetan egonkorrak diren gelatinazko egiturak sortzeko aukera eskaintzeaz gain, independenteak diren faseak egitura bakarrean mihiztatzeko metodologia baliagarria izan zitekeelaren hipotesia osatu genuen. Horretaz gain, entzimatikoki erretikulatutako 3D gelatinazko hidrogelen barnean zelulak kapsularatzeko aukera izateak, hidrogel anisotropoak prestatzeko proposatutako beste teknologia batzuekin erlazionatutako muga garrantzitsuak gainditu ditzake.

Adibidez, sistema anisotropoak norabide-izozte teknika jarraituz ekoiztean erabilitako zelulen baldintza ezegokiak tarteko, zelulak egitura horietan eransteko aukera bakarra aurrez prestatutako sistemen gainean 2D ereintzara mugatzen da [57]. Alabaina, 3D hazkuntza zelularrarekin mikroingurune biokimiko eta biomekaniko errealagoak eta *in vitro* eginiko errendimendu biologikoaren ebaluazio zehatzagoak lortu daitezkeela frogatu izan da [58].

Lehenik eta behin, hidrogel monofasiko mineralizatuak eta anisotropoak garatu ziren, *in vitro* karakterizazioa egin eta errendimendu biologikoa kapsularatutako giza gantz-ehunetik eratorritako zelula-amekin (*human adipose derived stem cells* edo hASCs) ebaluatu genuen. Fase mineralizatua osatzeko, HA partikulak gehitu ziren gelatina sarean eta SEM irudiekin antzeman zen moduan (9a. Irudia), mikropartikulak ondo sakabanatu ziren entzimatikoki erretikulatutako gelatina hidrogelaren baitan.

Bestalde, egindako konpresiozko entsegu mekanikoen arabera, partikula biozeramikoak gehitzeak hidrogelen gogortasuna areagotzen dute. Horrela, mineralizatutako konpositeen Young modulua, gelatina hutsez osatutako hidrogelena baino bi aldiz handiagoa izan zen (9b. Irudia). Aipatzekoa da, sendotutako hidrogelen Young modulua osteoideen zurruntasunarekin bat etorri zirela. Osteoblastoek jariatutako hezur-ehunaren aitzindaria den kolageno erretikulatuari deritzo osteoide [59].

Jarraian, mineralizatutako sistemek kapsularatutako hASCen desberdintzapen osteogenikoa bideratzeko zuten gaitasuna aztertzeko asmoz, alde batetik jariatutako ALP aktibitatea eta bestalde, hezurrarekin erlazionaturiko osteopontina (*osteopontin* edo OPN) adierazlearen espresio maila immunotindaketa bidez ebaluatu zen [60]. Mineralizatutako hidrogelean kapsularatutako hASC zelulek jariatutako ALP aktibitatean antzemandako (9c. Irudia) gutxinakako handiagotzearekin eta OPN adierazpen maila nabariagoekin (9d,e. Irudia), zelula horien desberdintzapen osteogenikoa sustatzeko formulazio horren berezko gaitasuna berretsi zen. Izan ere, markatzaile horren maila altuak hezur ehunaren birmoldatzearen arduradun diren zelula aitzindarien desberdintzapen osteogeniko berantiarrean gertaturiko ECMaren mineralizazio prozesuarekin lotzen dira [61].



9. Irudia. Hidrogel mineralizatuen karakterizazio fisikoa eta biologikoa. (a) Gelatina_hidroxiapatita (HA) konposite hidrogelen SEM irudi adierazgarria. (b) Hidrogelek konpresio entseguan lortutako Young moduluaren balioak. Esanahia estatistikoa: **** $p<0.0001$. (c) hASC zelulek jariatutako fosfatasa alkalinoaren (*alkaline phosphatase* edo ALP) aktibitatea hazkuntzan 1, 2 eta 3 aste mantendu ondoren. Hazkuntza denbora bakoitzean taldeen arteko desberdintasun estatistikoak * $p<0.05$ eta **** $p<0.0001$ moduan irudikatuta. (d,e) Osteopontina (*osteopontin* edo OPN) (berdez) tindaketaren irudi konfokalak eta adierazpen mailaren kuantifikazioa hazkuntza 6 eta 21 egunez mantendu ondoren. Zelulen nukleoak urdinez eta zitoeskeletoa gorriz antzemanak. Esanahia estatistikoa: **** $p<0.0001$. Eskala= 75 μm.

Fase mineralizatuaren formulazioa optimizatu ondoren, antolaketa anisotropoa zuten hidrogel biobateragarriak garatzea izan zen gure helburua. Ildo horretan, berebiziko garrantzia du tendoi eta lotailu ehun konektiboen bereizgarri diren ECMaren arkitektura ordenatua eta antolaketa zelularra mimetizatzea, lehenago aipatu bezala ezaugarri horiek funtsezko eginkizuna betetzen baitute ehun horien funtzio bai biomekanikoan eta baita biologikoan ere [57].

Horretarako, zelulosazko nanokristalak (*cellulose nanocrystals* edo CNC) gelatina hidrogelean barneratu ziren eta erretikulazio prozesua erlatiboki indar magnetiko txikiaren eraginpean burutu zen. Zelulosa mikrofibrilen eremu kristalinoetatik erauzitako hari formako nanopartikula horien erabilgarritasuna, ehunen ingeniaritzako hainbat aplikazioetan aurretik frogatu izan da. Tamaina nanometrikoko betegarri hauen ezaugarri garrantzitsuenetarikoen artean, jokabide mekaniko bikainak, biobateragarritasun egokia eta eremu magnetikoaren eraginpean orientazio aldaketarako sentsibilitatea aurkitzen dira [62]. Gure helburua urrats honetan, CNC partikulen izaera diamagnetikoa kontuan izanda, indar baxuko eremu magnetikoak erabiliz hidrogel matrizean euren orientazioa manipulatzea izan zen [63]. Hidrogel anisotropoak (AnisoGEL) prestatzeko CNC partikulen bi kontzentrazio (0,5 eta 1 % (m/b)) eta indar magnetikoaren bi maila (200 eta 400 mT) erabiltzea aztertu ziren. Kontrol gisa erabiltzeko hidrogel isotropoak (IsoGEL), gelatina/CNC konpositeak erretikulazioa eremu magnetikoaren esposizio gabe prestatu ziren. Eremu magnetikoaren indukziorako, neodimiozko bi iman iraunkorrez osatutako neurrira eginiko sistema magnetikoa erabili zen. Sistema horrekin aplikatutako eremu magnetikoaren indarra zehazteko gaussometro bat erabili zen. Aipatutako bi imanen arteko distantzia egokituz, euren artean kokaturiko hidrogelengan eremu magnetikoaren indar maila ezberdinak eragin daitezkeela frogatu zen [64].

Baldintza horiek berrantolaketa anisotropoa eragiteko nahikoa ote ziren ebaluatzeko, SEM bidezko mikroegituraren analisia, polarizatutako mikroskopio optikoaren bidezko birefrigentzia azterketa eta konpresio propietate mekanikoen ebaluazioa egin ziren. Nano osagaiez sendotutako gelatina sistemen barneko antolaketaren balorazioa liofilizatutako laginekin egin zen.

Kasu guztietan egitura porotsuak lortu baziren ere, alde batetik IsoGEL formulazioetan inolako antolaketa zehatzik gabeko ausaz orientatutako poroak antzeman ziren eta bestalde, AnisoGEL sistemetan poro handiagoak zituzten egitura antolatuagoak ikusi ziren. Efektu horren atzean, gelatina kateen berrantolaketa espaziala dago ziurrenik. SEM irudiekin eginiko norabide-analisien emaitzek taldeen arteko egiturazko desberdintasunak berretsi zituzten. Izan ere, AnisoGEL hidrogelen kasuan, aztertutako bi kontzentrazioetan orientazio angelu jakin batean maiztasun piko nabaria antzeman zen (10a-c. Irudia).

Antolaketa lerrokatuko hidrogelak lortzeko beharrezko eremu magnetikoaren esposizio denbora zehazteko asmoz, mikroskopio optiko polarizatua erabili zen. Horretarako, gelatina sareen erretikulazio fisikoa eragin zen denbora tarte ezberdinetan, laginak izotz bainuan murgilduz. Esposizio magnetikorik gabe prestatutako gelatinazko nanokonpositeekin, kolore anitzeko transmizio irudiak lortu ziren denbora tarte guztian. Emaitza horren arabera, birefrigentzia heterogenoa erakusten duten ausaz antolaturiko domeinu anitzeko egiturak lortu ziren. Alderantziz baina, AnisoGEL sistemen kasuan, irudi multi-kromatikoetatik abiatuz mono-kromatikoak ziren irudietara gertaturiko trantsizioa nabaritu zen, birefrigentzia homogenoa zuten domeinu bakarreko egituren formakuntza berretsiz [65].

Aipatutako trantsizio kromatikoa AnisoGEL lagin guztiekin 15 minuturen barruan antzeman bazen ere, CNCen kontzentrazioa handitzeak eta erangindako eremu magnetikoaren indarra areagotzeak, nanokristalen lerrokatze azkarragoa eragiten dute. Hori dela eta, partikulak %1-eko kontzentrazioan eta 400 mT-ko indarraren eraginpean prestaturiko hidrogelen (AnisoGEL1_400) kasuan, 10 minutu behar izan ziren irudi mono-kromatikoak lortzeko.

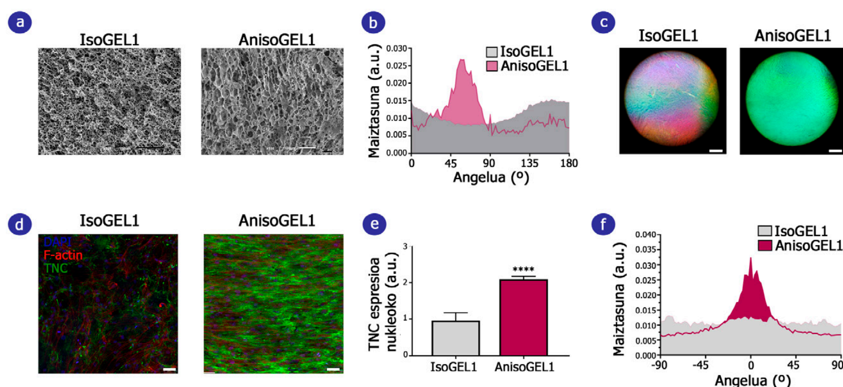
Emaitza hauek bat datoz aurrez bildutako literaturarekin, izan ere CNC suspentsioen hasierako ordenamendu azkarra minututan gerta daitekeela frogatu da, eremu magnetikoaren indarra areagotuz prozesua azkartzea lortzen delarik [66]. Kontuan izan behar da gainera, gure ikerlanean lortutako lerrokatzerako denbora tarteak egokiak direla diseinatutako hidrogelen erretikulazio entzimatikorako denborekin. Hidrogel horiekin hainbat ezaugarri erreologiko aztertu ziren eta erretikulazio entzimatikoa 20 minuturen bueltan gertatzen dela egiaztatu zen, ehunen ingeniaritzara bideratutako beste formulazio injektagarri batzuen antzera [67].

Horretaz gain, erretikulazio prozesua eremu magnetikoaren eraginpean prestaturiko nanokonposite hidrogelen kasuan mikroegitura anisotropoaren presentzia, konpresio mekanikoko ezaugarrien azterketarekin baieztatu zen. Nano tamainako betegarriak gelatinazko hidrogeletan gehitzeak efektu sendotzailea izatea frogatu zen, CNCen lerrokatzeak konpresio propietateen areagotzea eraginez gainera. Beste egile batzuk aurrez iradoki bezala, konpresio norabideari perpendikularki lerrokatuta antolatutako CNC zurrunen planoek, hidrogelaren konpresioarekiko erresistentzia areagotzea dakar [68].

Azterketa fisiko hauen ondoren, hidrogel anisotropoen berezko propietate nanotopografikoek zelulen portaeran eta desberdintzapen prozesuan izan zezaketen eragina ikertzeko, ehun anisotropoekin erlazionaturiko ezaugarri morfologikoak eta biokimikoak ebaluatu ziren. Aurretik deskribatu izan den moduan, seinale nanotopografikoen tamaina, geometria eta antolaketa espazialek zelula-amen hainbat prozesu biologikorengan eragina izan dezakete, hala nola zelulen atxikimenduan, migrazioan, proliferazioan, morfologian edota desberdintzapenean [64,69,70].

Efektu horiek mekanotransdukzio izenaz definituriko prozesuaren bitartez koordinatzen dira eta hainbat bide molekularren partehartzea frogatu da ECMetik zelulen nukleora bideratutako estimuluen transferentzian [69].

Gelatina eta CNC partikulekin osatutako hidrogeletan kapsularatutako hASCek bideragarritasun handia erakutsi zuten denboran zehar, kontzentrazio ezberdineko hidrogelen kasuan desberdintasun garrantzitsurik antzeman gabe. Ondorioz, garatutako gelatinazko konposite horiek zelulen 3D hazkuntza bermatzeko egokiak zirela ondorioztatu zen.



10. Irudia. Gelatina/zelulosa nanokristal konpositez osatutako hidrogelen erretikulazioan eremu magnetikoaren esposizioak duen eragina. (a) Ekorketazko mikroskopia elektroniko bidezko karakterizazio morfologikoa (eskala = 50 µm). (b) Hidrogel isotropo eta anisotropoen norabide-egituraren ebaluazioa. (c) Guztiz erretikulatutako hidrogelak mikroskopia optiko polarizatuarekin aztertu ondorengo irudiak. (d-f) Giza gantz-ehunetik eratorritako zelula-amen tenaszina (*tenascin* edo TNC) (berdez) deposizioa, zelulak 3D nanokonposite hidrogeletan kapsularatu eta 21 egunez kultibatu ondoren. Normalizatutako TNC adierazpen maila eta deposizioaren norabide-antolaketaren ebaluazioa. Eskala = 100 µm. Esanahia estatistikoa: **** $p<0.0001$.

Lerrokatutako hidrogelen egituraketak zelulen zitoeskeletoaren antolakuntzan izandako eragina ebaluatzeko, zelulak 3, 7 eta 21 egunez hidrogelen baitan kapsularatuta mantendu ondoren, F-aktinaren tindaketa egin zen eta zelulen orientazioa fluoreszentziazko mikroskopio konfokala erabiliz aztertu zen.

Aipatutako hidrogeletan barneratutako zelulek ardatz itxurako morfologia aurkeztu zuten hirugarren egunerako eta 3Dtan sakabanatutako zelulen dentsitate hazkuntzak denboran zeharreko proliferazioa gertatu izana iradoki zuen. IsoGEL sistemetan kapsularatutako zelulek lehentasunezko orientaziorik gabeko ausazko antolaketa erakutsi zuten bitartean, AnisoGEL formulazioko zelulek hirugarren egunetik aurrera antolaketa lerrokatua eta lehentasunezko orientazioa aurkeztu zuten, kultiboa aste betez izan ondoren efektua mantendu zelarik. Gainera, antolaketa eragin hori hidrogelen berezko ezaugarri anisotropoen ondorioz izan zela frogatu zen, orientatutako hazkuntza kultibo denbora osoan zehar baimentzeko

ezaugarri topografikoak nahikoak izan zirelarik, estimulazio magnetiko gehigarriren beharra izan gabe [64].

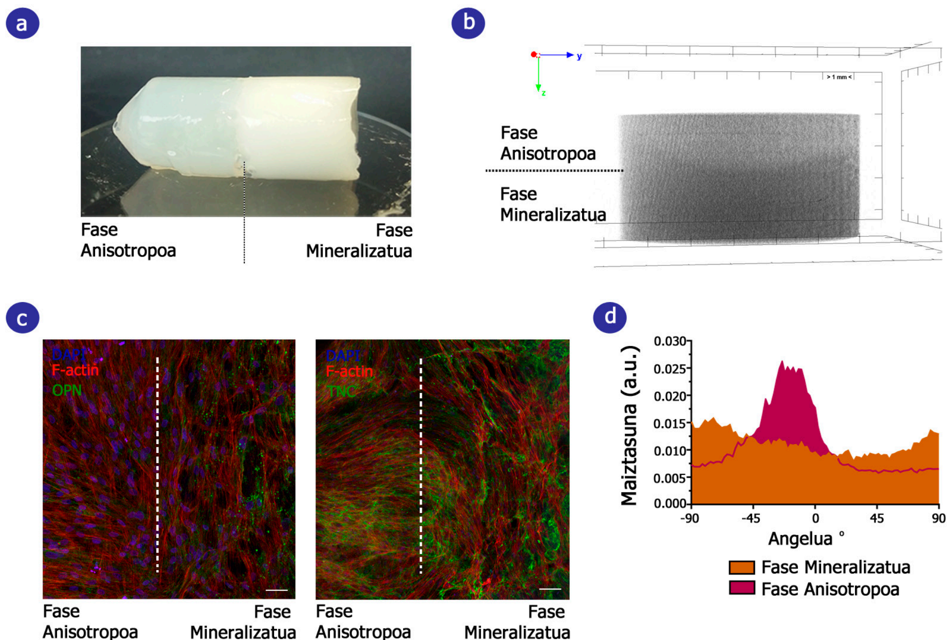
Bestalde, AnisoGEL1_400 hidrogeletan kapsularatutako hASC zelulak 21 egunez hazkuntzan mantendu ondoren, hidrogel isotropoetan barneratutakoen kasuan baino, nukleo luzatuagoak, tenaszina (*tenascin* edo TNC) espresio handiagoa eta glikoproteina honen zuntzezko deposizio antolatuagoa erakutsi zuten (10d-f. Irudia). Emaitza horien arabera, garatutako gelatinazko nanokonposite anisotropoek, hASC zelulak konpromiso tenogenikoruntz bideratzeko gaitasuna erakusten dute [71].

Hidrogel anisotropo horiek prestatzeko erabilitako eremu magnetikoaren indarrak zelulen antolaketan izan dezaketen indarrari dagokionez, ez zen desberdintasun garrantzitsurik antzeman 200 eta 400 mT-ko eraginpean sortutako hidrogelen artean. Hori dela eta, ikerlan honetan aztertutako eremu magnetiko altuek hidrogelen anisotropia fisikoa areagotu zuten arren, ezberdintasun hori ez zen nahikoa izan 3D zelulen antolakuntzan eragina izateko. Beraz, emaitzak modu orokorrean kontsideratuz, jatorrizko tendoi ehunen adierazgarri den ingurune anisotropoa simulatzea lortu genuen, magnetikoki sentikorrak diren gelatina/CNC konpositeak diseinatuz eta beraien egituraren moldatzea indar txikiko eremu magnetikoaren esposizioaz lortuz.

Jarraian, ezaugarri biomimetiko mineralizatuak eta anisotropoak hidrogel independenteetan ikertu ondoren, gure hurrengo helburua gelatina hidrogelen funtzionaltasun moldatuak 3D sistema bifasiko bakarrean integratzea izan zen. Alde batetik, gelatina/HA konpositea aukeratu zen hezur-ehuna simulatuko zuen fasea osatzeko eta bestalde, 400 mT-ko eremu magnetikoaren eraginpean erretikulatutako gelatina/CNC konpositea tendoi ehunaren egitura anisotropoa mimetizatu ahal izateko. Gelatina hidrogelen mihiztadura entzimatikoaz erabat elkartutako egiturak prestatzeko estrategiaren bideragarritasuna aztertzeko, bifasikoak ziren hidrogelak μ-CT teknikaren bitartez aztertu ziren. Laginaren azterketatik berreraikitako 3D irudiak erakutsi zuenez, ondo integratutako faseen arteko trantsizio leuna zuen egitura jarrai bakarra arrakastaz prestatzea lortu zen (11a,b. Irudia). Behaketa horren arabera, aurrez erretikulatutako gelatinazko hidrogelean erreakzionakorrak diren funtzio talde askeen presentzia dela eta, entzimaren aktibitate katalitikoari esker alboan hidrogel berri bat atxikitzeko aukera dago. Horrenbestez, fase anitzeko hidrogelen prestaketarako fabrikazio metodo oso sinplea eta erreproduzigarria iradoki genuen, integrazio helburuetarako aurrez proposaturiko prozedura konplexuen beharra saihestuz [72].

Behin hidrogel bifasikoak prestatzeko baldintzak zehaztu ondoren, fase bietan hASC zelulak kapsularatu genituen, egitura integratuan gelatina hidrogelen funtzionaltasun egokituak mantentzen ziren frogatzeko. Zelulaz beteriko 3D sistema 21 egunez kultibatu zen *in vitro* eta nukleoa, zitoeskeletoa, OPN eta TNC espezifikoki tindatu ziren. Fase mineralizatuan

ereindako zelulak lehentasunezko orientaziorik gabe ausaz sakabanatu ziren bitartean, fase anisotropoan zelulak modu lerrokatuan hazi ziren (11d. Irudia). Gainera, immunofluoreszentzia irudiek erakutsi zutenez OPN eta TNC deposizioetan gradienteak lortu ziren. OPN markatzaile osteogenikoaren espresioa HAz sendotutako hidrogelaren zatian nabariagoa izan zen einean, TNC deposizioa nabarmenagoa izan zen fase anisotropoan (11c. Irudia).



11. Irudia. Ehun-interfazeen ingeniaritzarako ezaugarri mineralizatuak eta anisotropoak integratzen dituzten hidrogel bifasikoak. (a) Faseak ondo bereizita dituen hidrogel bifasikoaren irudi digitala. (b) Hidrogel bifasikoen µ-CT azterketaren emaitzetatik berreraikitako 3D irudia. (c) Desberdintzapen osteogenikoaren markatzaile osteopontina (*osteopontin* edo OPN) eta tendoi ehunekin erlazionaturiko tenaszina (*tenascin* edo TNC) espresioaren azterketa, giza gantz-ehunetatik eratorritako zelula-amak (*human adipose-derived stem cells* edo hASCs) 21 egunez hidrogel bifasikoetan kultibatu ondoren. Eskala = 100 µm. (d) Bereizitako fase bakoitzeko zelulen zitoeskeletoen lerrokatze analisia.

Hortaz, gure emaitzek frogatu zuten, hidrogel bifasikoak garatzeko proposaturiko mihiztadura entzimatikoak konposiziozko gradienteak prestatzea ahalbidetzen duela, fase bakoitzeko egiturazko ezaugarriak eta kapsularatutako zelulen portaera biologikoarengan eragin gabe.

Gainera, hezur-tendoi interfaze ehunen sendaketa prozesua gehiago bultzatzeko asmoz, lan honetako gelatina eta mTG entziman oinarrituriko garapen estrategia are gehiago egokitu eta hobetu daitekeela aurreikusten dugu. Alde batetik, aipaturiko diseinuaren moldagarritasuna aprobetxatuz, fase ezberdin gehiago gehituz, neurrira eginiko fase anitzeko

sistemak garatu litezke. Kontzeptu froga moduan, ondo bereizitako hiru fase zituen skaffold-a prestatzea lortu genuen.

Bestalde, fase bakoitzean hazkuntza faktore espezifikoak kargatzeak zelula-amen desberdintzapena fenotipo zehatzetara bultzatzeko aukerak eskaini ditzake [9]. Hala ere, esan beharra dago oraindik beharrezkoak direla faktore biokimikoen konbinazio zehatzek zelula-amen desberdintzapen patroietan duten eraginari buruzko oinarrizko ikerketak burutzea.

Hobekuntza horiez gain, ikerlan honetan egindako azterketa biologikoak soilik hASC zelulak erabiliz egin baziren ere, bioinspiratutako 3D zelula heterotipikoen kultura plataformak erraz garatu daitezkeela uste dugu, proposaturiko faseetako bakoitzean zelula mota desberdinak kapsularatuz. Ildo horri dagokionez, gaur egun ikertzen ari diren hainbat ganberako gailuak oso interesgarriak izan litezke fase bakoitza hazkuntza medio espezifikoarekin kultibatu ahal izateko, zelula mota bakoitzak beharrezko mantenugai eta baldintzak eskainiz.

Laburbilduz, doktorego tesi honetan gelatinan eta entzimatikoki elkargurutzatzeko estrategian oinarrituz, hezur-ehunera eta hezur-ehunarekin erlazionaturiko interfazeen ehunen ingeniaritzara zuzendutako 3D plataforma egokituen garapenean jarri dugu arreta. Oro har, gure datuek medikuntza birsortzaile alorraren baitan biomaterial honen kontsolidazioarako aurrerapen garrantzitsua eskaini dute.

Erreferentzien zerrenda 200 -203 orrialdeetan aurkitzen da

Ondorioak

Doktorego Tesi honetako ikerketa esperimentaletan lortutako emaitzak oinarritzat harturik, honako ondorio hauek atera dira:

1. Transglutaminasa mikrobiarra entzima erabiliz, entzimatikoki erretikulatutako hiru dimentsiotako gelatinazko skaffold-ak arrakastaz garatu ziren liofilizazio teknikaren bitartez. *In vitro* eginiko karakterizazio azterketek erakutsi zuten, egitura horiek endotelio baskularraren hazkuntza faktorea (*vascular endothelial growth factor* edo VEGF) eta hezur proteina morfogenikoa-2 (*bone morphogenetic protein* – 2 edo BMP-2) hazkuntza faktoreak askatzeko gaitasuna izateaz gain, gainean ereindako zelulen hazkuntza bultzatu zutela ere.

2. Hidroxiapatita eta kaltzio sulfatoarekin sendotutako gelatinazko hiru dimentsiotako skaffold-ek, gizakien hezur-muinetik eratorritako zelula-amen desberdintzapen osteogenikoa induzitu zuten. Hezur proteina mofogenikoa-2 (*bone morphogenetic protein* – 2 edo BMP-2) hazkuntza faktoreaz funtzionalizatutako egiturek, sagu osteoporotikoen tamaina kritikoko garezur-gangako akatsen hezur-birsorkuntza bultzatu zuten.

3. Mikroegitura anisotropoa duten gelatinan oinarritutako hidrogelak arrakastaz garatu ziren zelulosazko nanokristalak formulazioan barneratuz eta erretikulazio prozesua indar txikiko eremu magnetiko uniformearen eraginpean burutuz. Hiru dimentsiotako sistema horietan kapsularatutako giza gantz-ehunetik eratorritako zelula-amen hazkuntza eta tendoi ehunarekin erlazionatuta dagoen tenaszina glikoproteinaren deposizioa lerrokatua izan zen.

4. Gelatinan oinarritutako hidrogel bifasikoak fase mineralizatu eta anisotropoaren mihiztadura entzimatikoaren bidez garatu ziren. Gelatina/hidroxipatita eta gelatina/zelulosazko nanokristalez osatutako konpositeek, hezur eta tendoi ehuna mimetizatzeko egokitutako ezaugarriak eskaini zituzten, hurrenez hurren.