

UNIVERSITÀ DEGLI STUDI DI SALERNO

Dipartimento di Medicina, Chirurgia ed Odontoiatria



Dottorato di Ricerca in
Medicina Traslazionale dello Sviluppo e dell'Invecchiamento Attivo
XXXIV ciclo

Curriculum: Medicina Traslazionale e Clinica

Tesi di Dottorato

OPTIMIZATION OF THREE DIMENSIONAL SCAFFOLDS AND GROWTH FACTOR CONTROLLED DELIVERY FOR CARTILAGE TISSUE ENGINEERING

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Anno Accademico 2020/2021

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"



UNIONE EUROPEA
Fondo Sociale Europeo



*Ministero dell'Università
e della Ricerca*



Abstract

Articular cartilage lesions cannot spontaneously heal because of the lack of vascularization and low metabolic activity of resident chondrocytes making clinical management difficult. Indeed, current therapies are restricted only to counteract inflammatory symptoms without promoting cartilage healing, and tissue engineering could represent a valid treatment option in patients with these disorders. However, to date, cell therapies are still not curative and have several technical issues, especially for cell harvesting and *in vitro* expansion before transplantation to the injury site. For example, a proper scaffold choice for *in vitro* cultures is essential for effective cell proliferation and differentiation, and collagen-based biomaterials are the best supports for hosting chondrocytes or stem cells, because of their similarities to natural extracellular matrix (ECM). Therefore, collagen-based scaffolds are being investigated as a valid alternative for *in vitro* chondrogenic commitment of human stem cells for further clinical use. In this thesis work, we aimed to develop a novel bioengineered three-dimensional (3D) scaffold for induction and maintenance of chondrogenic phenotype by seeding human bone marrow-derived mesenchymal stem cells (hBM-MSCs) in a type I collagen hydrogel functionalized along with biopolymeric microcarriers for providing a controlled release of transforming growth factor $\beta 1$ (TGF- $\beta 1$), a well-established chondrogenic growth factor. First, the appropriate TGF- $\beta 1$ concentration in culture medium for successful chondrogenic induction of hBM-MSCs was investigated using standard monolayer culture approaches. Cells cultured using a medium supplemented with 10 ng/mL of TGF- $\beta 1$ showed higher chondrogenesis-related genes (*SOX9*, 4-fold; *COL2A1*, 2-fold) and protein expression (type II collagen, 2.74-fold) after 16 days, and cells showed a prevalent expression of pro-inflammatory cytokines (*IL-12A*, 6-fold). Once optimized culture conditions, a 3D high-density approach was employed for chondrogenic commitment of hBM-MSCs, because standard 2D cultures promote cell de-differentiation and a switch toward a fibroblastic phenotype. 3D high-density spheroids were assembled using hBM-

derived or Wharton's Jelly (WJ)-derived MSCs, an alternative source of stem cells. This culture condition promoted a more marked upregulation of all chondrogenic genes and proteins compared to the 2D approach, probably because of synergic effects between TGF- β 1 delivery and high-density culture promoting cell-to-cell contacts. Moreover, we showed that WJ-MSCs might represent a good alternative for 3D models in cartilage tissue engineering. Next, hBM-MSCs were cultured in a collagen hydrogel functionalized with poly-lactic-co-glycolic acid microcarriers (PLGA-MCs) loaded with TGF- β 1 to provide a 3D biomimetic environment for chondrogenic commitment of stem cells. Cells were cultured under static and dynamic conditions using a perfusion bioreactor, and chondrogenic commitment was studied showing that chondrogenesis was induced only under perfusion conditions (*COL2A1*, 5-fold). Finally, to improve mechanical properties and enzymatic resistance of collagen hydrogel, we synthesized a novel composite scaffold using an equine type I collagen and hyaluronic acid under a reaction in heterogeneous phase.

In conclusions, our proposed data open new perspectives for clinical uses of collagen-based scaffolds and PLGA carriers as regenerative tools for cartilage lesion healing.

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Abbreviations

2D	Two- dimensional
3D	Three-dimensional
ACAN	Aggrecan
ACI	Autolog chondrocytes implantation
ALP	Alkaline phosphatase
AOC	Allophycocyanin
ATR	Attenuated total reflection
BMI	Body mass index
BSA	Bovine serum albumin
CHO-K1	Chinese hamster ovary cells
COL1A1	Type I collagen
COL2A1	Type II collagen
COL3A1	Type III collagen
COMP	Cartilage oligomeric matrix protein
COX-2	Cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
DSDs	Droplet size distributions
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK-1	Extracellular signal-regulated kinase-1
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FDA	Food and drug administration
FE-SEM	Field emission electron scanning microscopy
FITC	Fluorescein isothiocyanate
FT-IR	Fourier transform infrared spectroscopy

GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hyaluronic acid
hAD-MSCs	Human adipose-derived mesenchymal stem cells
hASC	Human adipose stem cells
HA-TBA	Tetrabutylammonium salt of hyaluronic acid
hBM-MSCs	Human bone marrow derived mesenchymal stem cells
hGDF-5	Human growth differentiation factor 5
hPBMCs	Human peripheral blood mononuclear cells
hTGF- β 1	Human transforming growth factor β 1
hWJ-MSCs	Human Wharton's Jelly-derived mesenchymal stem cell
ICRS	International Cartilage Repair Society of cartilage
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-10	Interleukin 10
IL-12A	Interleukin 12A
IL-1 β	Interleukin 1 β
IL-6	Interleukin 6
iPSCs	Induced pluripotent stem cells
JNK	C-Jun N-terminal kinase
MAP	Mitogen activated protein
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Human mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
N-CAM	Neural cell adhesion molecule
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
Pax 1	Paired Box 1
PBS	Phosphate-buffered saline
PC7	Phycoerythrin cyanin 7
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin

PFA	Paraformaldehyde
PGSS	Particles from Gas Saturated Solution
PIC	Polyion complexes
PLA	Poly lactic acid
PLGA	Poly-lactic-co-glycolic acid
PLGA-MCs	Poly-lactic-co-glycolic acid microcarriers
PMMA	Poly-methyl methacrylate
PSDs	Particle Size distributions
PSR	Picrosirius red staining
q-IF	Semiquantitative immunofluorescence
RH	Relative humidity
Rod-B	Rhodamine B
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX-2	Runt-related transcription factor 2
SAS	Supercritical Anti-solvent
SCX	Scleraxis
SD	Standard deviation
SE	Solvent evaporation
SEE	Supercritical emulsion extraction
SF	Shrinkage factor
SOX9	SRY-Box transcription factor 9
TE	Tissue engineering
TGA	Thermogravimetric analyses
TNF- α	Tumor necrosis factor α
VLE	Vapour Liquid Equilibrium
WJ	Whorton's Jelly
α -MEM	Minimum essential medium alpha

Publications and presentations

Lamparelli, E.P., Palazzo, I., Ciardulli, M.C., Scala, P., Reverchon, E., Forsyth, N.R., Maffulli, N., Santoro, A., Della Porta, G. *Supercritical emulsion extraction fabricated PLA/PLGA micro/nano carriers for growth factor delivery: Release profiles and cytotoxicity*. International Journal of Pharmaceutics. 2021; 592, 120108.

Lamparelli, E.P., Lovecchio, J., Ciardulli, M.C., Giudice, V., Dale, T.P., Selleri, C., Forsyth, N.R., Giordano, E., Maffulli, N., Della Porta, G. *Chondrogenic Commitment of Human Bone Marrow Mesenchymal Stem Cells in a Perfused Collagen Hydrogel Functionalized with hTGF- β 1-Releasing PLGA Microcarrier*. Pharmaceutics. 2021; 13(3):399.

Govoni, M., Lamparelli, E.P., Ciardulli, M.C., Santoro, A., Oliviero, A., Palazzo, I., Reverchon, E., Vivarelli, L., Maso, A., Storni, E., Donati, M.E., Ruspaggiari, G., Maffulli, N., Fini, M., Della Porta, G., Dallari, D. *Demineralized bone matrix paste formulated with biomimetic PLGA microcarriers for the vancomycin hydrochloride controlled delivery: Release profile, cytotoxicity and efficacy against S. aureus*. International Journal of Pharmaceutics. 2020; 582, 119322.

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Lamparelli, E.P., Ciardulli, M.C., Scala, P., Scognamiglio, MR., Charlier, B., Di Pietro, P., Izzo, V., Vecchione, C., Maffulli, N., Della Porta, G. *Thyroid hormone encapsulation in lipid nano-vesicles: comparison between different fabrication technologies, drug loading and in vitro delivery to human tendon progenitor stem cells*. International Journal of Pharmaceutics 2022 (**under revision**)

Lamparelli, E.P., Casagrande, V., Pressato, D., Maffulli, N., Della Porta, G., Bellini D. *Synthesis and Characterization of a Novel Composite Scaffold based on Hyaluronic Acid and Equine Type I Collagen for Cartilage defects*. Frontiers in materials 2022 (**under revision**)

Oral presentations and poster

“Espressione di un fenotipo condrale da parte di cellule staminali mesenchimali umane da midollo grazie ad un microambiente bioingegnerizzato 3D di collagene/PLGA a rilascio controllato di TGF- β 1 coltivato in perfusione” 3° Congresso Nazionale IDBM: “3D Printing and Bioprinting in Medicine and Surgery”, 28-30th, October 2019, Pisa (IT). **(oral presentation)**

“3D collagen system for TGF- β 1 controlled delivery and stem cells commitment towards chondrogenic phenotype” 9° I.S.M.u.L.T. Congress: Open Mind and New Technologies in muscles, ligaments and tendons, 29-30th, November 2019, Verona (IT). **(oral presentation)**

“PLA/PLGA nanocarriers designed for tissue engineering application and fabricated by Supercritical Emulsion Extraction” YoungInnovation: the state of research communicated by young researchers-Arising new manufacturing technologies NanoInnovation 2020 15-18th, September 2020, Roma (IT). **(poster and oral presentation)**

“Biopolymeric carriers developed for tissue engineering application and fabricated by Supercritical Emulsion Extraction” 18th European meeting on supercritical fluids, 4th-6th May 2021, Talence (FR) **(oral presentation)**

“Engineered outer membrane vesicles from E. coli: a platform for immunotherapy and drug delivery systems” FEBS 2021, 45th FEBS Congress 3-8th July, Ljubljana, Slovenia **(poster presentation)**

“Polymeric nanocarriers to control growth factors release within 3D scaffolds for tissue engineering” 13th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Rotterdam, 28th -31st March 2022 **(poster presentation accepted)**

In vitro model for chondrogenesis: 3D high density culture vs collagen 3D scaffold with growth factor controlled 10° I.S.MuL.T. Congress. “Open mind and new technologies in muscles, ligaments and tendons”. Roma, 8th – 9th April 2022 (**oral presentation accepted**)

Acknowledgments

At the end of my course of study, I would like to sincerely thank all people who contributed to achieving this important objective.

First of all, special thanks go to my two academic supervisors, prof. Giovanna Della Porta and prof. Nicola Maffulli, for all of their help in supporting me with this project, as well as for their essential contribution to my job training. Their generous support is invaluable, and I am so grateful for the time, expertise, and encouragement they gave to me.

I am very grateful to my industrial tutor Dr. Davide Bellini and all his staff, including Dr. Veronica Casagrande, Dr. Nicolò Morellato, and Dr. Daniele Pressato who gave me valuable advice and for their availability and kindness during my industrial secondment at Novagenit srl.

In addition, thanks to Prof. Carmine Selleri and my dear friend Dr. Valentina Giudice of Hematology and Transplant Center University Hospital “San Giovanni di Dio e Ruggi d’Aragona”, for human stem cell supply and their characterization.

For the design and development of the perfusion bioreactor, I would like to thank Prof. Emanuele Domenico Giordano and Dr. Joseph Lovecchio of Department of Electrical, Electronic and Information “Guglielmo Marconi” at the University of Bologna.

Furthermore, I would like to thank Prof. Ernesto Reverchon and his supercritical fluids group of Industrial Engineering Department of University of Salerno, above all Dr. Mariarosa Scognamiglio and Dr. Ida Palazzo for their contribution to microcarrier fabrication and material characterization.

A special thank goes to Prof. Nicholas Robert Forsyth and Dr. Tina Patricia Dale, of Guy Hilton Research Center at Keele University, for precious revisions of my scientific data and contribution to microscopy analysis.

Finally, special thanks go to two people: my colleagues Dr. Maria Camilla Ciardulli and Dr. Pasqualina Scala, with whom I shared these enjoyable three years.

**CHAPTER 1: Cartilage tissue engineering:
a new therapeutic weapon against osteoarthritis**

1.1 Cartilage within the human body: description, function, and classification

Human musculoskeletal system is a fully-fledged apparatus providing shape, support, and kinetic capability to the body ¹, and consists of different organs and tissues, such as bones, muscles, tendons, ligaments, and cartilage, working together in a coordinated way to ensure a correct posture, full mobility of limbs, and protection of vital organs ². Cartilage is an important structural component of the body and is present in different areas, including ears, nose, bronchial tubes, spine, and epiphysis of bones, and is a highly specialized connective tissue characterized by elasticity and mechanical strength. Within the joint, its principal task is to absorb shock reducing friction between bones, thus preserving their integrity ³. This tissue is composed of two types of specialized cells: chondrocytes, mature cells with poor mitotic potential, also involved in the mineralization process of unhealthy cartilage, and their precursors, chondroblasts, producing an extracellular matrix rich in collagen fibers and proteoglycans ⁴. It should be noted that chondrocytes, under specific environmental conditions, can return to a chondroblastic phase and resume their synthetic activity ⁵. Cartilage does not have either blood and lymphatic vessels, or nerves, therefore nutrient and oxygen uptake occurs through passive diffusion by near tissues. This process is significantly influenced by the permeability of extracellular matrix. Moreover, the lack of direct vascularization contributes to making cartilage a tissue with very low renewal capability ⁶. Cartilage is usually protected by a dense connective fibrous tissue, known as perichondrium, rich in fibroblasts that produce a matrix based on type I collagen, involved in protecting and supplying nutrients. This shell structure is absent in articular cartilage, where instead tissue is in direct contact with synovial liquid ⁷. Cartilage tissue, according to matrix chemical composition and resulting mechanical features, is classified into three types: elastic, fibrous, and hyaline cartilage ⁸ (see **Table 1**). This classification is in accordance with the International Cartilage Repair Society of cartilage (ICRS) standard evaluation ⁹.

Table 1 Characteristics of different cartilage tissues within the body

	Elastic cartilage	Fibrous cartilage	Hyaline cartilage
Cell types	Chondroblasts Chondrocytes	Fibroblasts Chondrocytes	Chondroblasts Chondrocytes
Extracellular matrix	Type II collagen Aggrecan	Type I and II collagen	Type II collagen Aggrecan Chondroitin sulfate
Perichondrium	Present	Not present	Present, except in joints
Functions	Structural support	Weight-bearing	Reduces friction
Main locations	Ears and epiglottis	Intervertebral discs	Synovial joints

1.1.1 Elastic cartilage

Elastic cartilage, also known as yellow cartilage owing to its staining, is a structural tissue for body parts that are not subjected to load forces, including auricle, eustachian tube, and epiglottis ¹⁰. Consequently, its damage is not very common compared to the other types present in the body. From a biochemical point of view, its composition is similar to hyaline cartilage but contains a most widespread network of flexible fibres, mainly elastin and fibrillin, interacting with each other inside a solid matrix ¹¹. The presence of these fibres allows elastic cartilage to have shape memory, reverting easily to its original configuration after being mechanically deformed. To observe elastin fibres, in histology, Verhoeff's stain is used, which exploits their affinity for the hematoxylin-iron complex, giving them a black colour ¹². Elastic cartilage is avascular and contains a perichondrium as a coating, which is made up of two layers: an outer layer riching in fibroblasts and an inner one that contains chondroblasts, which are necessary for the synthesis of the extracellular matrix ¹³.

1.1.2 Fibrous cartilage

Among cartilage tissues, the fibrous one is the most robust and is primarily located in the intervertebral discs and in insertions of ligaments and tendons to bones, and is the only one containing type I collagen in addition to the common components of connective tissue, i.e proteoglycan and non-collagenous proteins ¹⁴. Type I collagen fibres provide tensile strength and in histology can be

highlighted with picrosirius red staining (PSR), particularly under polarized light owing to their birefringence properties. Using this type of microscopy is also possible to discriminate between type I and III collagen fibres ¹⁵. Despite fibrocartilage being a strong and dense tissue, it presents lower biomechanical features than hyaline cartilage. Therefore, when generating fibrous tissue within an articular cartilage lesion, the prognosis is never favourable for patients. As will be described below, the formation of fibrous tissue within cartilage lesions is one of the most relevant problems associated with surgical treatment of these disorders.

1.1.3 Hyaline cartilage and its peculiar extracellular matrix

As it is well-known, hyaline cartilage plays an important role in early stages of embryonic development, being involved in the osteogenesis process ¹⁶. Indeed, skeleton formation at this level can take place in two ways: the first mode, termed intramembranous ossification, is the direct conversion of mesenchymal tissue into bone and mainly leads to the development of flat bones of the skull ¹⁷. On the contrary, the second way involves hyaline cartilage tissue as a precursor and its subsequent evolution in bone tissue. This process is known as endochondral ossification and can be split into different phases ¹⁸. First, mesenchymal cells become aggregates due to the release of paracrine factors, leading to the expression of several transcription factors, including paired Box (*Pax 1*) and scleraxis (*SCX*) ¹⁹. Subsequently, these cell nodules become more compact and differentiate into chondrocytes following the expression of *SOX9* gene, a transcription factor involved in the early phase of chondrogenesis. *SOX9* together with *SOX5* and *SOX6*, the other two DNA-binding proteins, forms a complex known as the *SOX* trio involved in the chondrogenesis enhancement ²⁰. N-cadherin, a transmembrane protein expressed in different tissues with the function to promote adhesion among cells, plays a key role in condensation of mesenchymal stem cells ²¹, while neural cell adhesion molecule (N-CAM) is involved in the maintenance of cell aggregates over time. This complex process

has inspired the development of 3D high-density culture as an *in-vitro* approach for inducing chondrogenesis of human stem cells, as we will see in **Chapter 2**. The extracellular matrix of hyaline cartilage represents the released product from resident cells within each tissue and is composed of a mixture of structural and functional proteins, arranged in specific 3D textures ²². These molecules provide the mechanical force required for the proper function of each tissue. Specifically, the ECM of hyaline cartilage is composed of water, collagen (mainly, type II), proteoglycans (such as aggrecan), glycosaminoglycan (especially chondroitin and keratan sulfate, as well as hyaluronic acid), and a small fraction of other types of protein. These components help to retain water within the matrix, which is critical in maintaining its particular mechanical properties. Indeed, water is the most abundant component within ECM of articular cartilage, constituting about 80% of its wet weight ²³. The ECM of articular cartilage has a layered architecture, within which is possible to identify at least four distinct zones: superficial, middle, deep, and calcified areas different in terms of ultrastructure and composition (see **Figure 1**). The superficial zone is in contact with the synovial liquid and presents the smallest thickness among all the layers and consists mostly of type II collagen fibers aligned in a parallel manner to the articular surface for giving resistance to shear forces ²⁴. This zone also functions as a barrier, shielding cartilage from the immune cells coming from synovial fluid. Chondrocytes in this zone have flattened morphology and synthesize high concentrations of collagen (mainly type I and XI) and a low amount of proteoglycans; moreover, this zone has the highest water content and is covered by a clear film of small collagen fibrils and by a cellular layer of chondrocytes known as the “lamina splendens” ²⁵. This layout provides a smooth and scrolling surface to the tissue. Breaks in the superficial zone results in alteration of biomechanical features of cartilage, the first degenerative changes in early phases of osteoarthritis, especially in grade I lesions ²⁶. In contrast to the superficial zone, the transitional one shows lower cell density. It is mainly made up of round-shaped cells, embedded in an abundant extracellular matrix. Collagen fibers are organized obliquely and there is a higher proteoglycan concentration. This area allows a transition between the shearing

forces of superficial layer and the compressive forces in deeper zones. The deep zone in contact with bones consists of the same fibers perpendicularly aligned to the bone surface for distributing and absorbing compressive loads ²⁷. Finally, the calcified zone is anchored to the subchondral bone by hydroxyapatite crystals. This layer provides a barrier to diffusion from blood vessels supplying the bone. It contains a small number of cells embedded in a calcified matrix demonstrating a very low metabolic activity. Chondrocyte percentage within mature articular tissue is very low and occupies around 5% of the entire tissue volume. For this reason, hyaline cartilage has become an ideal candidate for regenerative medicine.

The architecture of hyaline cartilage

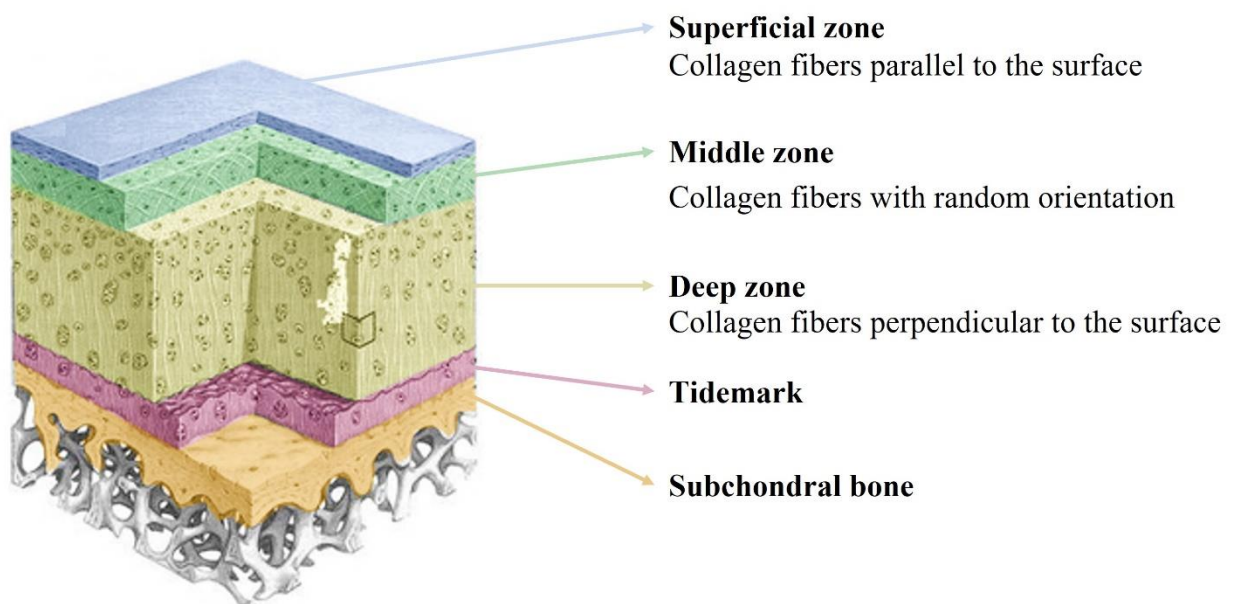


Figure 1 Schematic illustration of hyaline cartilage architecture on epiphysis of long bones.

1.1.4 Synovial Joints

The human body contains approximately 270 bones at birth, which later decrease over time due to the fusion of some of them as we age. Hundreds of joints connect bones to each other, thus allowing different degrees and types of movement to the body ²⁸. In more detail, human joints are classified into three groups according to movements that can allow: synarthroses, amphiarthroses, and

diarthroses ²⁹. Synarthroses are fixed joints and therefore do not allow any movement between bones, a practical example are sutures of the skull. On the contrary, amphiarthroses allow only slight motions and are mainly located in the spinal column. Finally, the most complex joints in terms of structure are diarthroses (also known as synovial joints), like those of the knee and shoulder. These last ones allow full mobility of bones but are extremely susceptible to wear processes. In more detail, synovial joints contain three structures: synovial cavity, joint capsule, and articular cartilage. The first one is a characteristic interspace between bones that hosts a viscous and non-Newtonian liquid, termed synovia. The main task of this fluid is to reduce friction between the cartilaginous surfaces during movement ³⁰. Furthermore, it has rheopectic properties, becoming more viscous only under shear pressure applied. The inner layer of synovial joints is termed synovial membrane and produces and releases synovial fluid within the joint cavity ³¹. Chemically, the synovial liquid is a dialyzate of blood plasma (ultrafiltrate) that contains high amounts of lubricin and hyaluronic acid. This last one is secreted by fibroblast-like cells in the synovial membrane, while lubricin (termed also as proteoglycan 4; PRG4) by the surface chondrocytes of the articular cartilage ³². Synovium may show significant changes in terms of chemical composition, even before visible cartilage degeneration has occurred, with infiltration of mononuclear cells, thickening of the synovial lining layer and production of inflammatory cytokines ³³. Therefore, the analysis of its composition may be useful to early diagnosis of articular disorders.

1.1.5 Osteoarthritis

Articular cartilage lesions can be induced by traumatic incidents, as in the case of sports and occupational accidents, or be a consequence of autoimmune diseases (e.g. rheumatoid arthritis). Regardless of their origin, these lesions can have an adverse course, leading to the development of a most complex chronic disease, referred to as osteoarthritis ³⁴. As previously mentioned, the possibility

of spontaneous remission is highly unlikely, both owing to insufficient vascularization of cartilage tissue and low metabolic activity of resident chondrocytes. In fact, this condition does not allow either the trigger of the fibrotic process or proper recruitment of immune cells in the injury site. Therefore, when cartilage damage is diagnosed, timely and effective clinical treatment is necessary to avoid an unfavorable prognosis. OA is a chronic degenerative disease involving joints, characterized by structural deterioration of hyaline cartilage, which leads to wear of underlying bones ²⁶ (see **Figure 2**). It is accompanied by several symptoms, ranging from pain and stiffness of the joint up to completely blocking of its mobility ³⁵. As is known, depending on the lesion morphology, a classification with different deterioration grades was drawn up. This ranking, developed by Outerbridge, assigns a grade of 0 through IV to the chondral area of interest, where 0 is associated with normal cartilage, while IV to most severe injuries involving also sub-chondral bone ³⁶. To date, the best methods for diagnosing and monitoring OA are represented by MRI and radiography. Moreover, X-rays are also useful to identify osteophytes that are pathological excrescences considered a further pathognomonic sign of osteoarthritis.

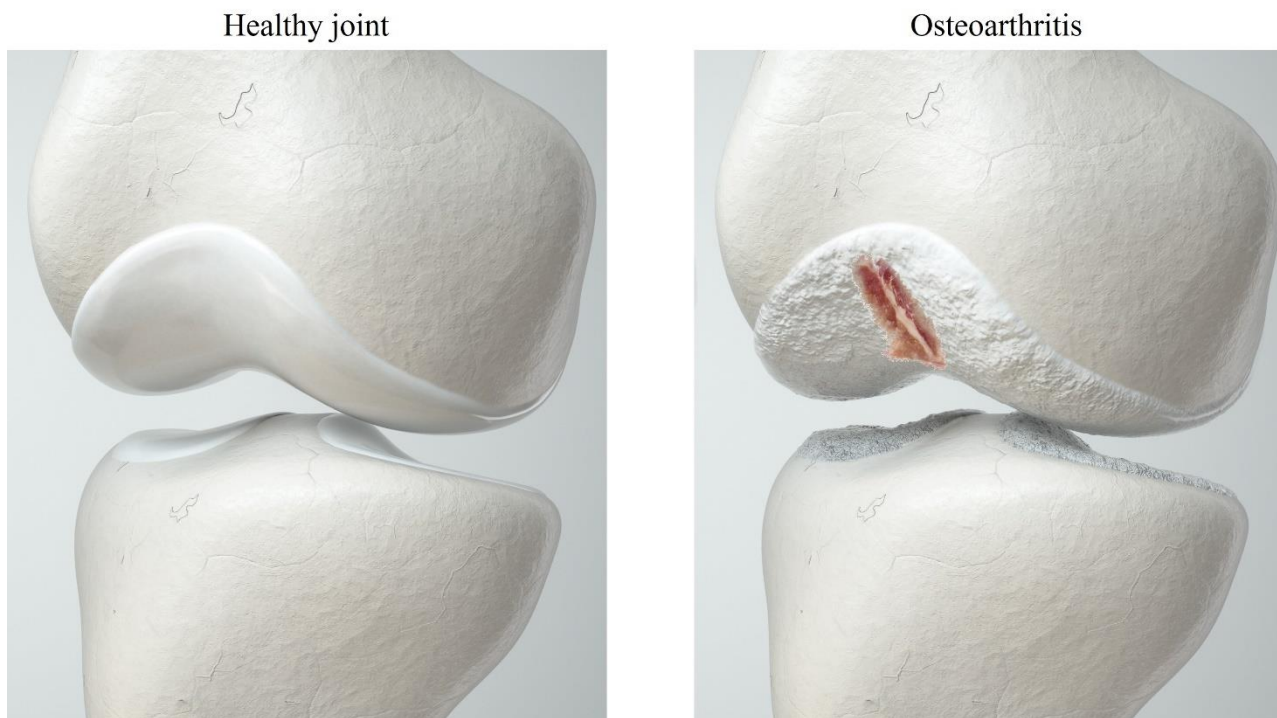


Figure 2 Comparison between healthy and pathological joints.

Osteoarthritis is characterized by degradation of hyaline cartilage on epiphysis of bones, thickening of the subchondral bone, and osteophytes formation.

Table 2 Outerbridge classification for joint cartilage lesions

Outerbridge classification	Description
Grade 0	Normal cartilage
Grade I	Cartilage with softening and swelling
Grade II	Cartilage surface damage (fibrillation and fragmentation)
Grade III	Deep cartilage damage without bone exposure
Grade IV	Erosion of cartilage down to bone

1.1.6 Pharmacological therapies

Clinical management of articular cartilage injuries remains a major challenge owing to the avascular and hypocellular nature of the tissue itself, which prevents their spontaneous remission. On the other hand, joint suffering associated with osteoarthritis is often disabling and interests a significant percentage of the aging population, impairing seriously the quality of their life over time. Currently, available treatments can be divided into conservative (including pharmacotherapy, arthrocentesis, and physiotherapy) and surgical ³⁷. The objectives of pharmacological treatment are mainly aimed to

relieve pain and inflammatory symptoms for decreasing functional disability as well as for avoiding complications during the prognosis of the disease. Unfortunately, until now no drug has been able to promote cartilage healing or regeneration. Moreover, non-pharmacological approaches are paramount, especially in elderly patients who traditionally already taking many drugs and have comorbidity. They consist of the acquisition of healthy eating regimes, physiotherapy sessions, and weight loss. The benefits of this last one can be significant because the BMI affects the pressure that the body exerts on the joints. In pharmacological approach, prescribed drugs are primarily anti-inflammatory and analgesic drugs, that effectively reduce local inflammation and pain.³⁸ Among NSAIDs drugs, acetaminophen is the most frequently prescribed being considered safer than others. Despite it seems to be effective in the short term in patients with pain from mild to moderate, its exact role in OA treatment pain is not defined yet³⁹. This drug is preferable to other NSAIDs, especially in the case of renal insufficiency. If sorrow does not improve following paracetamol treatment, it is possible to select another NSAID or even opioid. The efficacy of NSAIDs has been associated with their inhibitor effect on COX-2, which is upregulated by some cytokines, including IL-1 β and TNF- α , during OA⁴⁰. An alternative to NSAIDs is represented by intraarticular glucocorticoid injections. Despite seeming to relieve inflammatory symptoms as NSAIDs, pharmacological effect appears too limited in time, and, they also have several adverse effects, such as inhibition of chondrocyte proliferation⁴¹. Also hyaluronic acid is approved for the management of OA owing to its viscoelastic properties that enhance the lubrication within the joints⁴². On the other hand, this polysaccharide is secreted physiologically by synoviocytes into the synovial fluid. However, being approved as medical device it has not undergone the same intense efficacy check required for drugs approval, therefore, its clinical role is a bit controversial.

1.1.7 Surgical treatment

Patients who poorly respond to pharmacology therapies are ultimately treated with surgical procedures, such as arthroscopic debridement and lavage to alleviate symptoms associated with progression of articular deterioration ⁴³. Despite arthroscopy turning surgery from invasive to mini-invasive, studies related to clinical outcome of these procedures are weak and often inconsistent. Along with debridement, there are numerous surgical methods utilized for the management of articular cartilage defects with the aim of providing symptomatic relief and improving mobility. For instance, arthroplasty is one option that has long been employed in the treatment of joint diseases, especially for hip and knee, with very satisfactory outcomes. However, in younger and active patients, arthroplasty is a less satisfactory surgical option ⁴⁴. Non-arthroplasty options can be classified as reparative procedures (Microfractures) and regenerative procedures (Autologous Chondrocyte Implantation). Microfracture is a technique widely used for recruiting autologous MSCs within a cartilage lesion. This technique employs subchondral drilling to start cartilage repair by inducing bleeding, enabling mesenchymal cells to migrate from the bone marrow to the injury place. After this operation, the repaired tissue is primarily composed of fibrocartilage that has poorer biochemical and biomechanical characteristics than those of hyaline cartilage⁴⁵.

Among regenerative procedures, ACI (see **Figure 3**) has been adopted in different clinical contexts to repair cartilage damage. ACI is performed by biopsy of a small portion of cartilage tissue from joints by arthroscopy, and chondrocytes are enzymatically isolated, expanded using *in vitro* cultures, and then re-implanted at the injury site ⁴⁶.

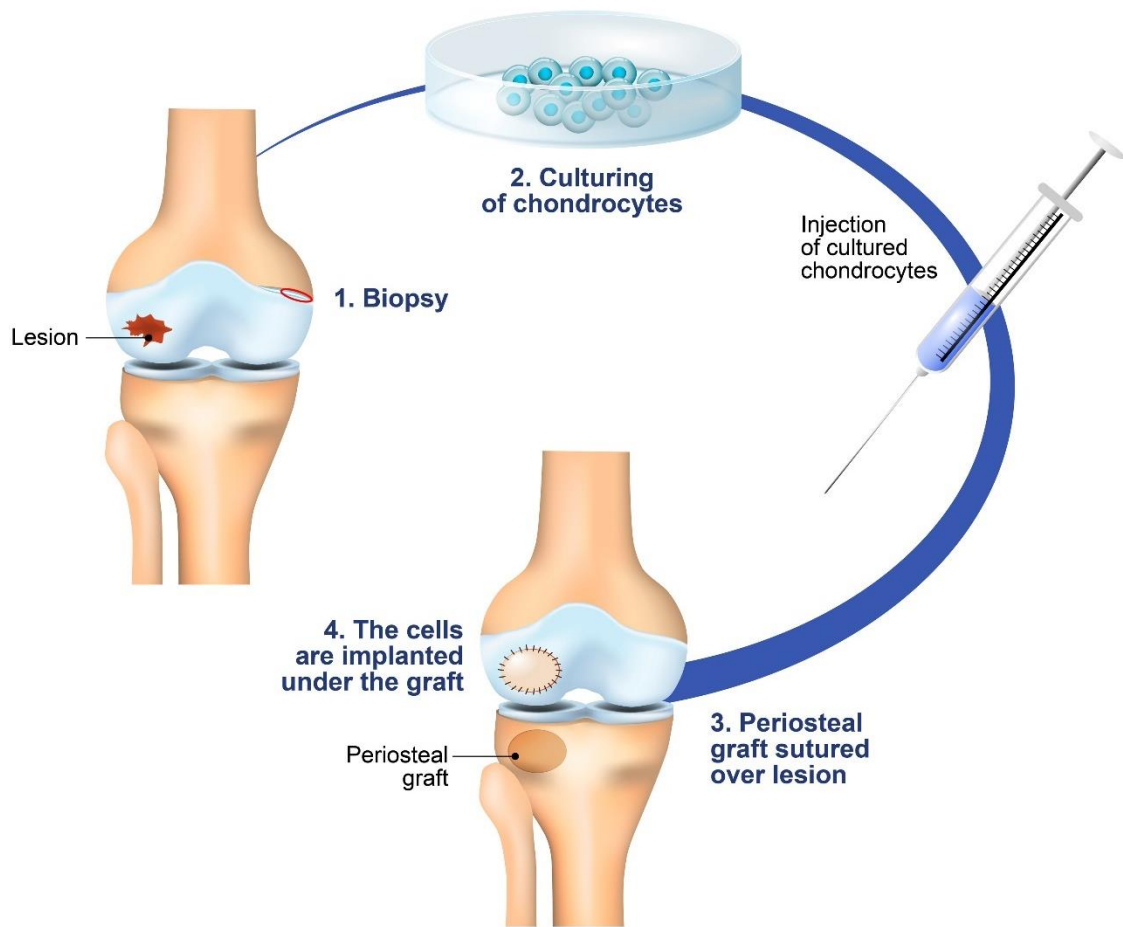


Figure 3 Schematic illustration of autologous chondrocyte implantation (ACI) procedures.

1.1 Cartilage tissue engineering

Clinical management of articular cartilage lesions represents one of the main challenges for modern orthopedics, especially owing to the failure of current treatments. Indeed, current approaches, based on pharmacological therapies and surgical procedures, are not yet able to promote the healing process and arrest cartilage structural deterioration. No medications are effective in terms of regeneration for patients suffering from OA, and surgical options are effective only in the short term because as time goes lead to the formation of tissue with different biomechanical properties such as fibrous cartilage. An innovative and promising therapeutic approach is tissue engineering, whose classical paradigm is

the combination of cells, scaffold, and chemical or mechanical signals (see **Figure 4**) with the purpose to manufacture biological substituents that will be finally inserted in the damaged site to guide its regeneration ⁴⁷. Therefore, tissue engineering is an alternative approach to traditional ones and offers great potential to improve joint cartilage lesions therapy. In more detail, it consists in implanting interactive and bioengineered supportable to stimulate the formation of a new cartilaginous tissue indistinguishable from the native one. In recent years, tissue engineering techniques have been described the literature to promote stem cells differentiation toward a chondrogenic phenotype. These techniques involve the use of 3D scaffolds, made of biocompatible and biodegradable polymers, able to host stem cells and growth factors in order to generate a bioengineered tissue that is immunologically, functionally, and structurally like to the natural one for obtaining a stable and lasting *restitutio ad integrum*. Current research is focused on improving mechanical and biochemical properties of scaffolds, to make them more similar to natural extracellular matrix. Materials employed as scaffolds must comply with strict requirements in order to be adopted in clinical settings, including biocompatibility, biodegradability as well as low immunogenicity ⁴⁸. Along with safety, these biomaterials must possess suitable mechanical properties to avoid their structural degradation once in vivo implanted. Therefore, the proper choice of biomaterials and human stem cells, as well as chemical and mechanical stimuli, is a crucial element for the success of tissue engineering.

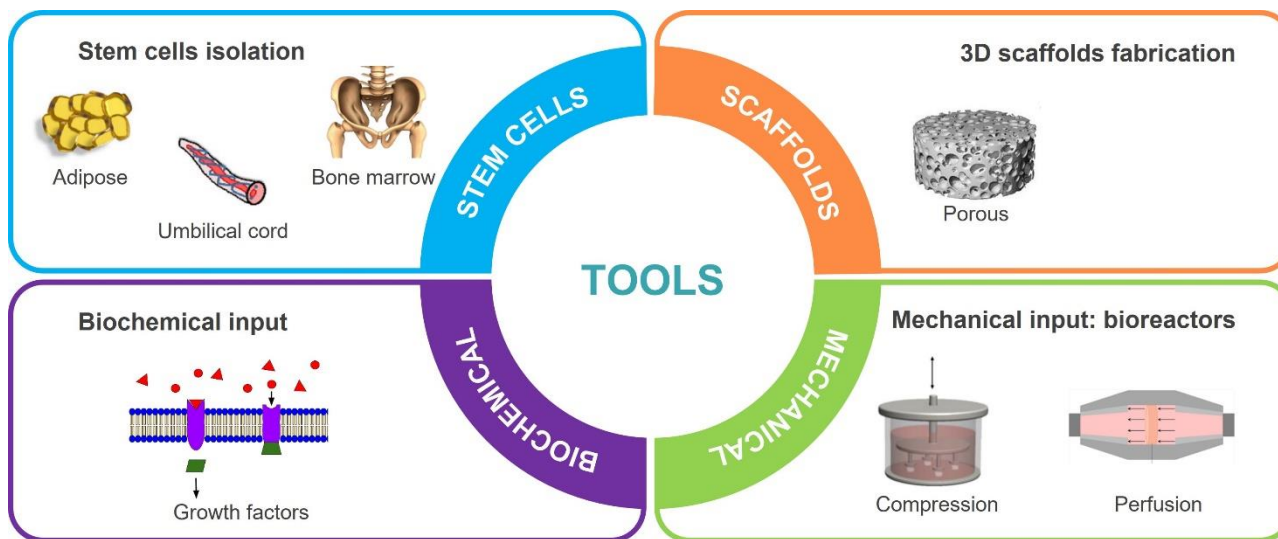


Figure 4 Representation of main tools used in tissue engineering protocols.

1.3.1 Stem cells

In the last few years, the use of human stem cells to promote healing of damaged tissues has raised a great deal of hope, especially in the contest of cartilage lesions. As previously described, autologous chondrocyte implantation can be used in the treatment of focal cartilage injuries to prevent the onset of OA. However, issues associated with chondrocyte harvest and expansion have encouraged to search for alternative solutions, including human stem cells ⁴⁹, that can be classified according to origins and ability to differentiate toward specific lineages. Considering this last criterium, stem cells can be divided into three categories: totipotent, pluripotent, and multipotent cells ⁵⁰. The first ones are capable to generate all tissues of the organism including extra-embryonic ones (i.e., placenta and umbilical cord), necessary for its development. For instance, blastomeres, resulting from the first divisions of the zygote, are considered the only totipotent stem cells ⁵¹. In contrast, pluripotent stem cells, such as ESCs and iPSCs, can give rise to all tissue types of the body, except extra-embryonic ones. Despite the great potential of iPSCs for regenerative medicine, they still show genetic instability during in vitro expansion, accumulating chromosomal abnormalities ⁵². Finally, Multipotent stem cells are cells that have the self-renew capability as well as to differentiate into multiple lineages

present in a specific tissue or organ. These cells show a limited capacity to differentiate with respect to totipotent and pluripotent cells. However, MSCs are the most interesting candidates in regenerative medicine and their use could be useful in the repair and regeneration of skeletal disorders through several mechanisms including differentiation and response to inflammatory conditions⁵³. Currently, the most studied sources of MSC are BM, adipose tissue, and WJ. These cells are potentially capable of differentiating into osteoblasts, chondrocytes, fibroblasts, and myocytes in vitro. Moreover, MSCs obtained from various sources have different abilities to secrete many different paracrine factors such as cytokines and growth factors. It is worthy of note that the proliferation rate of pluripotent cells is higher than multipotent ones, making them faster during in vitro expansion.

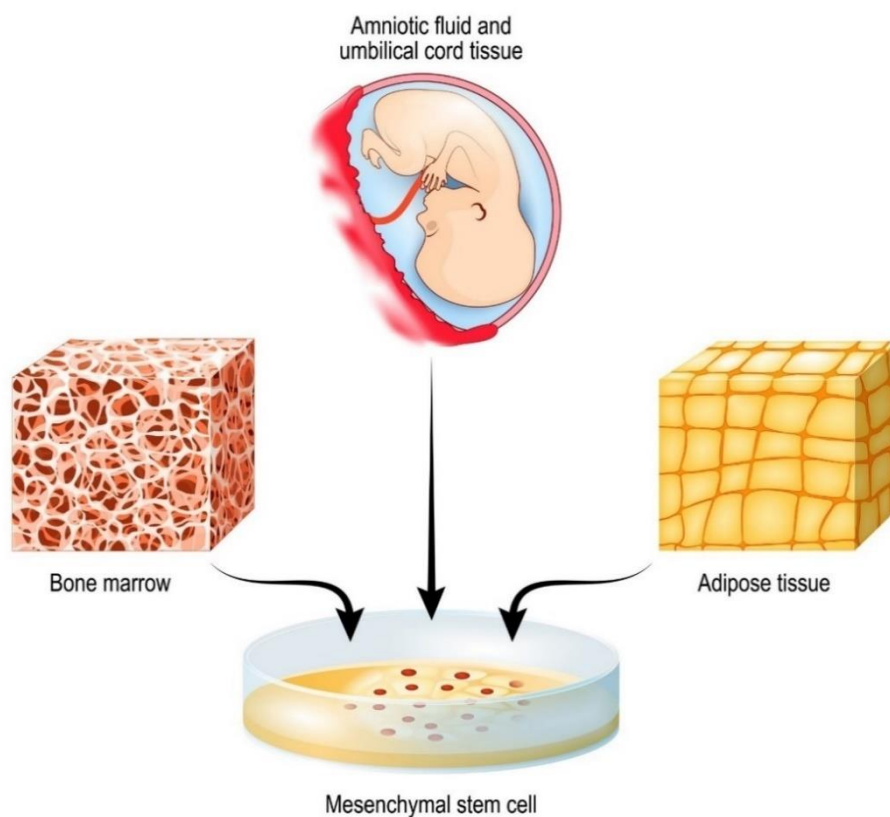


Figure 5 Schematic illustration of different sources of mesenchymal stem cells, including bone marrow, adipose tissue, and Wharton's Jelly.

1.3.2 Biochemical input: growth factors

Different methods to stimulate chondrogenic differentiation of MSCs *in vitro* and *in vivo* have been developed, like the addition of growth factors free or encapsulated in biopolymeric carriers to promote their controlled local release ⁵⁴. Peptides commonly used as chondrogenic inducers are those belonging to TGF- β superfamily, especially TGF- β 1 and TGF- β 3, routinely applied *in vivo* and *in vitro* studies for promoting chondrogenesis of different multipotent stem cells ⁵⁵. For TGF- β 1, a dose-response effect has been also described and the most effective concentration is 10 ng/mL, as already confirmed by previous investigations ⁵⁶. TGF- β 1 treatment of MSCs results in downregulation of type I collagen expression, which is considered a histological marker associated with fibroblast phenotype ⁵⁷. However, TGF- β 1 has a short half-life as a supplement in culture medium and microencapsulation has been proposed, utilizing biopolymeric vehicles, as a way to overcome this ^{54,58}. TGF- β 1 promotes chondrogenesis through activation of different intracellular signaling pathways including MAP kinases, p38, ERK-1, and to a lesser extent, JNK ⁵⁹. Another important aspect to consider is that although TGF- β stimulation is indispensable for promoting stem cells chondrogenesis, its prolonged interaction with receptors could promote overtimes hypertrophic maturation of MSC, limiting their clinical effectiveness. It is worthy of note that to improve the chondrogenic effect of growth factors, several studies suggest dexamethasone supplementation (at about 100 nM) to the culture medium ⁶⁰.

1.3.3 Mechanical input: bioreactors

Chondrogenesis of stem cells is traditionally induced by chemical stimulation, using different soluble growth factors that can be added in suitable concentration to the culture medium and stimulate cell receptors. However, also mechanical input has a critical role in inducing chondrogenic differentiation, as cartilage tissue is always subjected to load forces, starting from its development. Mechanical forces can be various, but stimuli associated with compression and perfusion seem to be more effective in chondrogenesis contexts ⁶¹.

In our experience, dynamic conditions significantly improve 3D culture systems⁶². In more detail, perfusion upregulates chondrogenic markers⁶³, preserves chondrogenic phenotype, and prevents hypertrophy of MSC-derived chondrocytes, a issue in cell-based strategies⁶⁴. Moreover, chondrocytes respond to mechanical loading by increasing the production of ECM components, including COMP, type II and IX collagen, and GAG⁶⁵. The instruments utilized for giving in vitro mechanical stimulation to cells are called bioreactors and are essential tools, especially in 3D culture approaches. Indeed, 3D cellular systems are characterized by a low mass exchange of nutrients as well as waste that affect cell viability. Therefore, the development of perfusion bioreactors represented a good solution for overcoming this issue

1.3.4 Scaffolds as synthetic extracellular matrices

In tissue engineering protocols, scaffolds are designed to give a comfortable three-dimensional environment with suitable features to support stem cells in their differentiation toward functional tissue of interest. Typically, this is achieved using biodegradable and biocompatible materials, which may be of natural or synthetic origin. The choice of the scaffold is crucial to the success of the therapeutic strategy. Here there is a brief description of these, even if the topic will be deepened in next chapters.

1.3.4.1 Collagen

Collagen is the most common structural protein of mammals and mainly lies in connective tissues, such as skin, tendons, ligaments, and cartilage. So far, over twenty types of collagen have been identified and they all show a typical triple helix structure⁶⁶. In tissue engineering, collagen-based scaffolds, are theoretically more appropriate to support the chondrocyte or stem cells attachment, because are more similar to natural ECM, in addition, are also biocompatible and biodegradable⁶⁷.

The collagen can be used in a different form, but hydrogel seems more suitable to distribute stem cells and growth factors; the only drawback is represented by lower mechanical strength which hampers the dynamic culture. Even although there are many types of collagens, only a few are used to produce biomaterials, particularly, type I collagen is currently the gold standard in the field of regenerative medicine, because is a suitable for implantation and only a small amount of people possess humoral immunity against it; the common sources of collagen include bovine skin and tendons, porcine skin and rat tail. Collagen-based scaffolds can be crosslinked in order to enhance their mechanical and enzymatic resistance properties for implantation purposes. Glutaraldehyde is the most employed chemical reagent used to crosslink collagen-based biomaterials, even if, genipin, a chemical compound derived from a vegetal source, shows an interesting potential to replace glutaraldehyde because of its low toxicity⁶⁸.

1.3.4.2 Alginates

Alginates are polysaccharides distributed widely in the cell walls of brown seaweeds. Chemically, they are linear copolymers of D-mannuronic acid (M) and L-guluronic acid (G). The gelation of these polysaccharides happens when blocks of monomer units bind with each other, thanks to the presence of divalent cations, usually calcium ions, promoting reticulation. The use of strontium or barium ions in place of calcium ions produces a stronger gel but reduces the biocompatibility of the scaffold⁶⁹. Alginates can be also modified to enhance some properties such as cell adhesion or the growth factors attachment⁶⁷. Alginate matrices provide a convenient means to preserve the chondrocyte phenotype during in vitro expansion, but their clinical application, as scaffold for cartilage repair, may be limited by the potential calcification of the constructs⁷⁰.

1.3.4.3 Chitosan

Chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl D-glucosamine residues, which derives from partially or fully deacetylation of chitin, a structural element of the fungal cell wall and the crustacean's exoskeleton. The deacetylation of chitin can be obtained with chemical or enzymatic treatments: the chemical treatment consists of alkaline hydrolysis while the enzymatic one, requires the use of particular enzymes, such as chitin deacetylases ⁷¹.

1.6 Thesis objective

The avascular and hypocellular nature of articular cartilage does not allow its spontaneous healing following injuries. Currently available treatments are still far from reaching tissue regeneration, being orientated mainly to symptomatic relief. Tissue engineering strategies could represent a turning point in treatment of articular lesions. The most effective clinical therapies involve the implantation of autologous chondrocytes into injuries site, leading to a significant clinical improvement. However, despite clinical success, there are still many issues associated with *in vitro* expansion and maintenance of chondrocytes. Therefore, further studies are necessary, also considering other cell types such as MSCs as a source of chondroblasts. The main objective of this PhD project was to develop 3D *in vitro* models of hyaline-like tissue for potential applications in articular cartilage healing and regeneration. The central hypothesis of this work was that a combination of collagen-based scaffolds, human stem cells, and PLGA-carriers loaded with growth factors, can be used as a platform to produce an *in vitro* physiologically relevant cartilaginous analogue.

**CHAPTER 2: Chondrogenesis of human stem cells:
from 2D monolayer to 3D high-density cultures**

2.1 Introduction

As described in Chapter 1, hyaline cartilage is a specialized connective tissue with the principal task to provide a regular and lubricated surface in joints, which helps to prevent the wear process ⁷² and minimizes subchondral bone pressure thanks to the distribution of load forces ⁷³. This tissue is composed of mature cells with poor mitotic potential, termed chondrocytes, and a complex ECM containing type II collagen and ACAN, the main histological cartilage markers ^{74,75}. Type II collagen fibers provide tensile strength, can be histologically highlighted with picosirius red staining, and are visible under polarized light because of their birefringence properties ¹⁵. Conversely, fibrous cartilage contains type I collagen and shows different biomechanical behavior ⁷⁶. Cartilage lesions represent a substantial challenge for modern orthopedics as they do not spontaneously heal due to the absence of vascularity and innervation and untreated lesions may have an unfavorable prognosis with the development of osteoarthritis ⁷⁷.

3D *in vitro* models of chondrogenic differentiation using human stem cells are becoming the most utilized model to study healing and regenerating events. Human MSCs derived from BM have a well-described chondrogenic potential ⁷⁸. MSCs, multipotent progenitor cells with self-renewal potential, can differentiate in several tissue types, such as muscle, adipose, and trabecular bone tissue; however, MSC harvesting from BM requires invasive procedures and the mesenchymal fraction is low ⁷⁹. For these reasons, alternative MSC sources have been identified, such as adipose and umbilical cord tissue comprised of WJ and umbilical cord blood ^{80,81}. However, hWJ-MSCs chondrogenic potential remains poorly investigated, despite those cells having a superior proliferative capacity which enables more rapid scaling and evaluation in experimental settings, making them extremely valuable in tissue engineering context ⁸². Moreover, MSCs could modulate microenvironment composition as described in some hematological disorders, such as myelodysplasia where they can contribute to hematopoietic stem cell growth inhibition by secretion of pro-inflammatory cytokines, especially TGF- β ⁸³.

Interestingly, TGF- β , both hTGF- β 1 and hTGF- β 3, is the principal growth factor used for routinely establishing chondrogenesis *in vitro* ⁵⁵. In particular, hTGF- β 1 is the most investigated, both *in vitro* and *in vivo* experiments ^{56,57}; however, hTGF- β 1 has a short half-life when supplemented in culture medium that might be overcome using growth factor microencapsulation with biopolymeric vehicles ^{54,58}. hTGF- β 1 promotes chondrogenesis through activation of different intracellular signaling pathways including MAP kinases, p38, ERK-1 and, to a lesser extent, JNK ⁵⁹. Monolayer cultures are considered unsuitable for both MSCs differentiation towards chondrogenic phenotype and chondrocytes expansion; furthermore, 2D culture is often reported to promote the dedifferentiation process of chondrocytes and the acquisition of fibroblast phenotype ⁸⁴.

Recently, stem cells 3D cultures were proposed for chondrogenesis study; indeed, this approach takes the advantage to better mimicking the cartilage microenvironment where chondrocytes reside. The two predominant strategies are either utilization of a biomaterial scaffold to support seeding and subsequent differentiation, ⁶², or alternatively, scaffold-free techniques, such as 3D high-density aggregate culture. This last approach has proven advantageous across a range of disease and tissue modeling scenarios ⁸⁵. These 3D-systems commonly display a hypoxic core which could promote chondrogenesis of stem cells. Moreover, the interactions with adjacent cells simulate those found in pre-cartilage condensations during embryonic development ⁸⁶. The production of these aggregates can follow a range of methods, even if the hanging drop technique has been reported to allow the control 3D-system size by modifying, for instance, the culture volume or cell density ⁸⁶.

Despite 3D cultures are well-recognized methods to study chondrogenic potential of various tissue derived MSCs, the ability of these stem cells in a 3D setting to modulate chondrogenic differentiation and cytokine production has not been reported yet

2.2 Aims

We investigated 3D high-density culture performance in promoting chondrogenic commitment of human stem cells, and in modification of cytokine expression during differentiation process. Specifically, BM and WJ-MSCs were used to produce 3D culture in the presence of hTGF- β 1. Chondrogenic commitment was evaluated by gene expression profiling and IHC analysis of specific markers, such as types I, II and III collagen, *SOX9* transcription factor and *ACAN*. Cytokine production was investigated by pro and anti-inflammatory cytokine gene expression during differentiation events.

2.3 Materials and Methods

2.3.1 hMSCs isolation, expansion, and characterization

hBM-MSCs were obtained from bone marrow (BM) of two healthy donors (males aged 38 and 40); whereas, hWJ-MSCs were isolated from the human umbilical cord of two donors (aged 23 and 31). Donors gave written informed consent in accordance with the Declaration of Helsinki to the use of their residual BM aspirate for research purposes, with approval from the University Hospital (Review Board prot./SCCE n. 24988 achieved on April 09th, 2015). Briefly, BM aspirate or umbilical cord were seeded in Minimum Essential Medium Alpha (α -MEM) supplemented with 1% GlutaGro™, 10% Fetal Bovine Serum (FBS), and 1% Penicillin/Streptomycin (Pen/Strep) and incubated at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity⁸⁷. After 72h, non-adherent cells and other residues were aspirated, fresh media added, and the remaining adherent cells fed with fresh media twice a week. On day 14, colonies of adherent MSCs were identified, detached, and re-seeded at 4000 cells/cm² in the same culture conditions. Once the cell culture reached 70–80% confluence, cells were detached using 0.05% trypsin-0.53 mM EDTA and washed with 1x phosphate buffered saline (PBS) (Corning Cellgro, Manassas, VA, USA), counted using Trypan Blue (Sigma-Aldrich, Milan, IT), and subcultured at a concentration of 4×10^3 cells/cm². At Passage 2, cells were used for the experiments.

Flow cytometry analysis was performed on both hBM-MSCs and hWJ-MSCs obtained at Passage 2 with antibodies directed against CD90, CD105, CD73, CD14, CD34, CD45, and HLA-DR expression (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germania), as better described elsewhere^{88–90}.

2.3.2 Flow cytometry and gating strategy for MSCs characterization

hWJ-MSCs were detached and counted; 1×10^5 cells were incubated at RT for 20 min with the following directly conjugated mouse-anti human antibodies: CD90 FITC (Beckman Coulter, Fullerton, CA, USA), CD73 APC (Miltenyi Biotec, Gladbach, Germany), CD105 PE (Beckman Coulter, Fullerton, CA, USA), CD45 PC7 (Beckman Coulter, Fullerton, CA, USA), HLA class-II FITC (Beckman Coulter, Fullerton, CA, USA), CD14 PC7 (Beckman Coulter, Fullerton, CA, USA) and CD34 PE (Beckman Coulter, Fullerton, CA, USA).

The isotype-matched immunoglobulins IgG1 FITC (Beckman Coulter, Fullerton, CA, USA), IgG1 PE (Beckman Coulter, Fullerton, CA, USA), IgG1 APC (Beckman Coulter, Fullerton, CA, USA), and IgG1 PC7 (Beckman Coulter, Fullerton, CA, USA) were used as negative controls under the same conditions. At least 15,000 total events were acquired with a BD FACSVerser flow cytometer (Becton Dickinson, BD, NJ, USA).

A minimum of 1×10^5 cells were stained for mesenchymal phenotype analysis. Manufacturer's instructions of used antibodies (Beckman Coulter) were optimized as follows. For antibody mix 1, 2.5 μ L of fluorescein isothiocyanate (FITC) - conjugated anti-CD90, 10 μ L of phycoerythrin (PE) - conjugated anti-CD105, 5 μ L of allophycocyanin (AOC) – conjugated anti-CD73, and 10 μ L of phycoerythrin cyanin 7 (PC7) – conjugated anti-CD45 antibodies were added. For antibody mix 2, 10 μ L of FITC – conjugated anti-HLA-DR, 10 μ L of PE – conjugated anti-CD34, and 10 μ L of PC7 – conjugated anti-CD14 antibodies were added. After 20 min incubation at room temperature,

samples were washed twice with phosphate buffered saline (PBS) and resuspended in the same buffer for acquisition.

FACSVerse cytometer (BD Biosciences) equipped with 2 lasers (blue, 488 nm, and red laser, 628 nm) and BD FACSuite software (BD Biosciences) were used for sample acquisition. Compensation was calculated using single-color controls for each fluorochrome and an unstained sample was used as negative control for setting PMT voltages. All samples were run using the same PMT voltages. A minimum of 30,000 events were recorded.

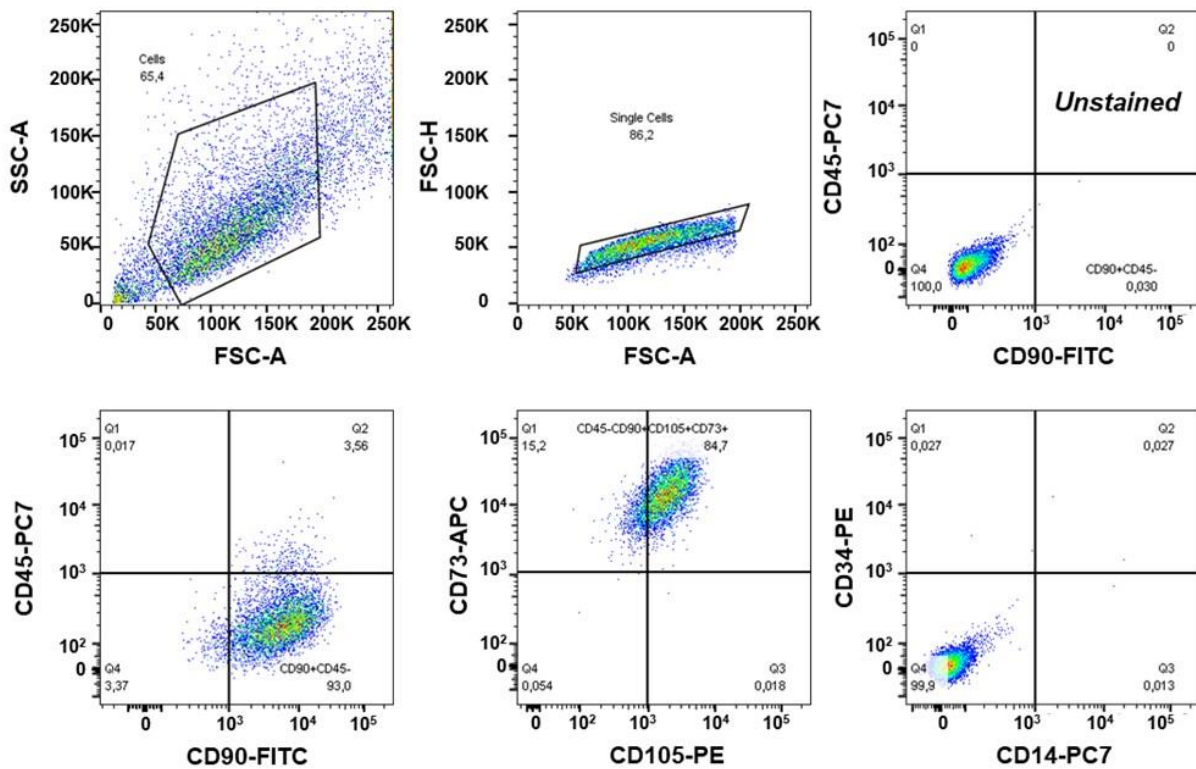


Figure 6 Representative flow cytometry analysis of WJ cells employed in our experiments.

2.3.3 hMSC culture

hBM-MSCs were seeded on coverslips at a concentration of 4×10^3 cells/cm². Once the cell cultures reached 60% confluence, cells were treated with chondrogenic medium and supplemented with either

1 ng/mL or 10 ng/mL of recombinant human TGF- β 1 (PeproTech EC, Ltd, London, UK). The chondrogenic medium was composed of alpha- minimum essential medium (α -MEM) (Corning, NY, USA) with reduced FBS to 1% (Corning Cellgro, Manassas, VA, USA) further supplemented with 1% ITS (Corning Cellgro, Manassas, VA, USA), 1% GlutagroTM (Corning Cellgro, Manassas, VA, USA), 50 μ M of ascorbic acid phosphate, and 1% Penicillin-Streptomycin. Cells were fed twice a week with fresh medium and growth factor for up to 16 days. Untreated cells for all the time-points studied served as control.

High density 3D cultures were obtained by the hanging drop method (**Figure 7**). Briefly, hMSCs were resuspended at a density of 500.000 cells/mL. Drops of the cell suspension (30 μ L) were dispensed on the lids of Petri dishes, which were then inverted. Hanging drop cultures were incubated and, after 3 days, the resulting cellular aggregates were harvested using a pipette and transferred into an ultra-low attachment 96 multi-well plate using 100 μ L of chondrogenic medium/well. The chondrogenic medium (supplemented with 10 ng/mL of hTGF- β 1) was changed every two days. 3D systems morphology was monitored with the ImageJ software (rel.1.52p National Institutes of Health, USA). The diameter, area (A) and perimeter (p) were measured, and circularity was calculated using the equation (1):

Equation 1

$$F \text{ circularity} = \frac{4\pi A}{p^2}$$

For the evaluation of Feret's diameter and circularity, the average value of 10 3D-systems ($n= 3$), were considered ⁹¹.

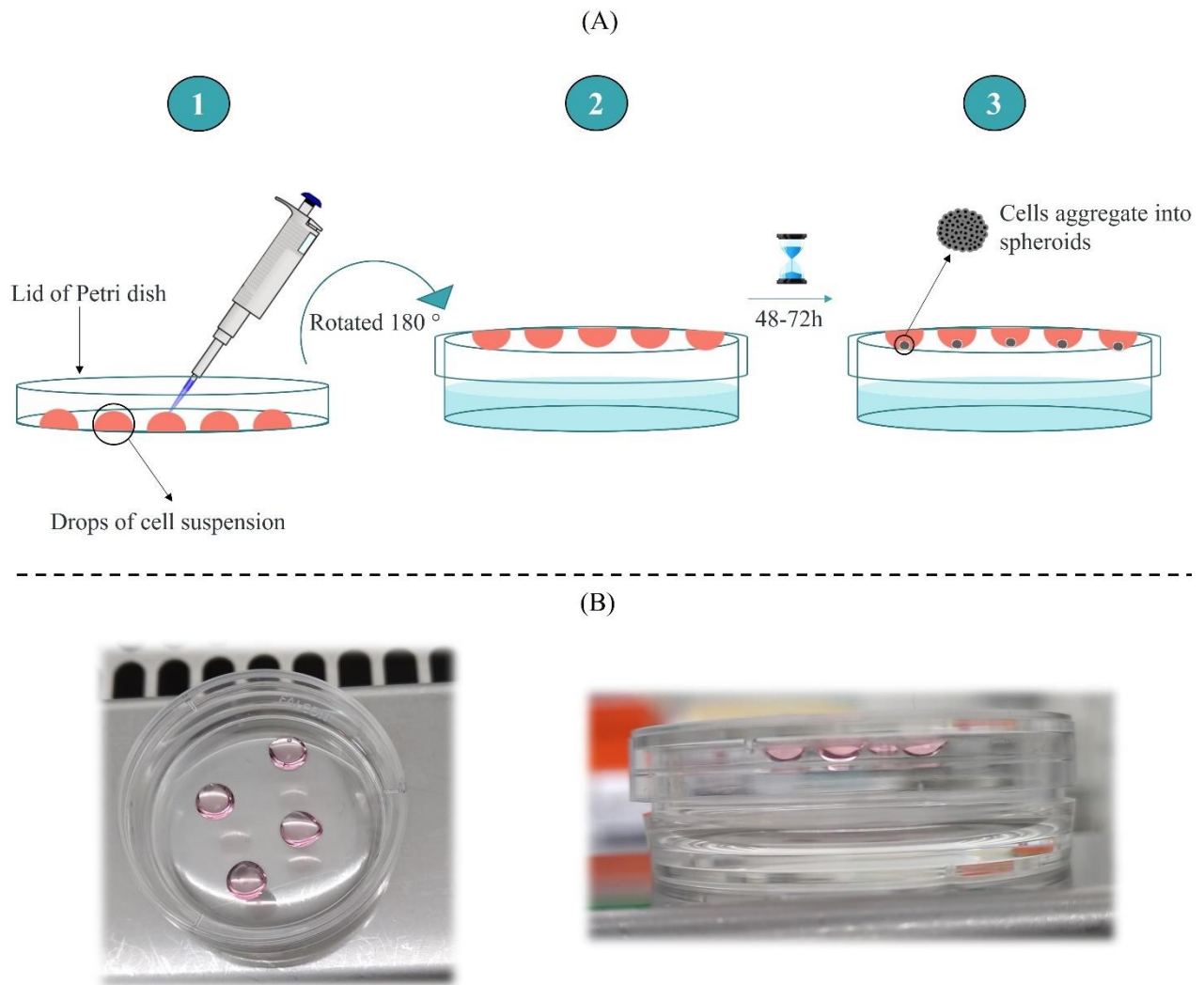


Figure 7 Schematic illustration and images of the hanging drop method.

Schematic illustration of different steps of the hanging drop method (a) for obtaining stem cells spheroids.

2.3.4 RNA isolation and gene expression profiling

Type II collagen (*COL2A1*), SRY-Related HMG-BOX Gene 9 (*SOX9*), Aggrecan (*ACAN*), type I, III and X collagen (*COL1A1*, *COL3A1* and *COL10A1*) markers were investigated, as well as pro-inflammatory cytokines Interleukin 6 (*IL-6*), Tumor Necrosis Factor α (*TNF- α*), Interleukin 12A (*IL-12A*), Interleukin 1 β (*IL-1 β*) and anti-inflammatory ones Interleukin 10 (*IL-10*) and *TGF- β 1*. Total RNA was extracted from hBM-MSCs at each time point using QIAzol[®] Lysis Reagent (Qiagen, DE), chloroform (Sigma-Aldrich, Milan, IT) and the Rneasy Mini Kit (Qiagen, DE). For each sample, 1

µg of total RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Milan, IT). Relative gene expression analysis was performed in a LightCycler® 480 Instrument (Roche, IT), using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Foster City, CA, USA) with the validated primers (Bio-Rad, Foster City, CA, USA) and following MIQE guidelines ⁹². Amplification was performed in a 10 µL final volume, including 2 ng of complementary DNA (cDNA) as template. Triplicate experiments were performed for each condition explored, and data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression ⁹³. Fold change in gene expression was determined by the $2^{-\Delta\Delta C_P}$ method and presented as relative levels vs. untreated cells at each time-point explored.

2.3.5 IHC assays

Cells were fixed with 3.7% paraformaldehyde (PFA) for 30 min at room temperature (RT), followed by permeabilization with 0.1% Triton X-100 for 5 min and blocked with bovine serum albumin (BSA) solution (1% w/v) for 1h. For type II and III collagen staining, cells were incubated overnight at 4°C with a rabbit polyclonal anti-type II collagen antibody (1:100, Cat no: ab34712, Abcam, Cambridge, UK) and a mouse polyclonal anti-type III collagen antibody (1:100, Cat no: sc166316, Santa Cruz Biotech., CA, USA). Then, cells were incubated for 1h at RT with the Alexa Fluor™ 488 goat anti-rabbit IgG (1:400, Thermo Fisher Scientific, Waltham, MA, USA) and the DyLight 649 anti-mouse IgG (1:500, BioLegend, San Diego, CA, USA) antibody. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using Leica laser-scanning confocal microscope (mod. TCS SP5; Leica Microsystems, Wetzlar DE) equipped with a plan Apo 63X/1.4 NA oil immersion objective. Signal intensity, related to the proteins of interest, was quantified using the software ImageJ (rel.1.52p National Institutes of Health, USA) ⁹⁴, when reported. Five images of

several fields were used for the analysis at each time point. All data were reported as fold change relative to untreated cells. Antibody specificity were assessed in our previous works^{62,89,95}.

3D-spheroids were fixed in 4% PFA for 2 h at room temperature, cryo-protected in 30% sucrose (4°C, overnight), included and sliced into 15 µm thickness samples using a cryostat (mod. CM1950, Leica, Wetzlar, Germany). For type I and II collagen staining, samples were incubated overnight at 4 °C with a mouse polyclonal anti-collagen type I antibody (1:100, Cat no: MAB3391, Abcam, Cambridge, UK) and rabbit polyclonal anti-collagen type II antibody (1:100, Cat no: ab34712, Abcam, Cambridge, UK). Following incubation with primary antibody, slices were incubated for 1h at RT with Alexa FluorTM 488 goat antirabbit IgG (1:400, Thermo Fisher Sci., Waltham, MA, USA) and DyLight 649 antimouse IgG (1:500, BioLegend, San Diego, CA, USA) antibodies. Cell nuclei were counterstained with DAPI.

2.3.6 Sirius Red Staining

For cartilage matrix histochemistry, a Sirius Red staining was performed as previously described^{96,97}. 3D-spheroids sections were stained in hematoxylin for 8 min, washed in water for 2 min, immersed into phosphomolybdic acid for 2 min, washed in water for 2 min, dipped into Picrosirius Red F3BA Stain (Polysciences, Inc., Warrington, PA, USA) for 60 min, and finally into HCl 0.1 M solution for 2 min. Samples were dehydrated with an increasing ethanol gradient (70%–75%–95%–100%) and cleared in xylene for 5 min. Finally, sections were mounted using Eukitt (Sigma-Aldrich, Milan, Italy) mounting medium. Picrosirius red brightfield and polarized light images were acquired with a Brunel polarization microscope equipped with a Nikon D500 camera.

2.3.7 Live and Dead staining

Cell viability within scaffolds was detected by fluorescence live/dead assay (Calcein AM solution (Cat. no C1359) and Ethidium homodimer I solution (Sigma-Aldrich, Milan, Italy). In more details, cells were stained for 1 h at 37 °C, washed in PBS 1×, and captured by fluorescence microscope (mod. Eclipse, Nikon Corporation, Tokyo, Japan). Single images were acquired with identical light intensity, exposure time, and gain settings. Signals intensity was quantified using ImageJ software (rel.1.52p National Institutes of Health, Bethesda, MD, USA). Original images in RGB format were converted into an 8-bit (gray scale) format, and tagged areas were expressed as an average value of pixel intensity within a range from 0 (dark) to 255 (white) as better described elsewhere ⁹⁸.

2.3.8 Statistical analysis

For comparison between two independent groups, a statistical analysis using the two-tailed independent Student's T test was performed. One-way or two-way ANOVA was used, followed by Tukey's multiple comparison test for the comparisons between more than two groups. P values <0.05 were accepted as significant ⁹⁹

2.4 Results

2.4.1 hTGF-β1 at 10 ng/mL efficiently induces chondrogenic differentiation of hBM-MSCs

To identify the optimal conditions for the 3D high-density culture system approach for chondrogenic commitment of hBM-MSCs, two concentrations of hTGF-β1 (1 and 10 ng/mL) were tested for induction of chondrogenic commitment of hBM-MSCs in a 16-day culture system. Higher concentration values were not considered following literature data describing an inhibitory effect ¹⁰⁰. Unsupplemented cells harvested at matched time points were used as negative control. Culture medium with 1 ng/mL hTGF-β1 resulted in a 2.33-fold increase ($p < 0.0001$) of SOX9 levels at day

16, whereas 10 ng/mL hTGF- β 1 stimulated a *SOX9* 3.94-fold up-regulation ($p < 0.001$). The lower hTGF- β 1 supplementation induced a marked *COL2A1* downregulation at day 8 followed by recovery of expression to control levels at day 16. In contrast, at 10 ng/mL concentration, *COL2A1* expression showed a 2-fold increased only at day 16. *ACAN* was downregulated compared to control at each time point at both hTGF- β 1 concentrations explored. Finally, *COL1A1* and *COL3A1* expression was always downregulated in response to hTGF- β 1 supplementation (**Figure 8A-B**).

Cytokines expression along the chondrogenic events was also monitored. Supplementation with 1 ng/mL hTGF- β 1 resulted in transcriptional upregulation of pro-inflammatory cytokines, especially *TNF- α* and *IL-12A*, respectively 2.25-fold ($p < 0.0001$) and 1.88-fold, at Day 8; while no changes in anti-inflammatory cytokines were described. After 16 days, only *IL-12A* retained significant upregulation (3.17-fold; $p < 0.01$) (**Figure 8C**). At 10 ng/mL, hTGF- β 1 supplementation promoted *TNF- α* (2.16-fold; $p < 0.01$) and *IL-12A* (1.8-fold; $p < 0.05$) upregulation after 8 days. Moreover, *IL-12A* (6.06-fold; $p < 0.01$) and *IL-1 β* (1.99-fold) displayed significant changes at Day 16. Among anti-inflammatory cytokines, only *IL-10* was upregulated (2.3-fold) (**Figure 8D**).

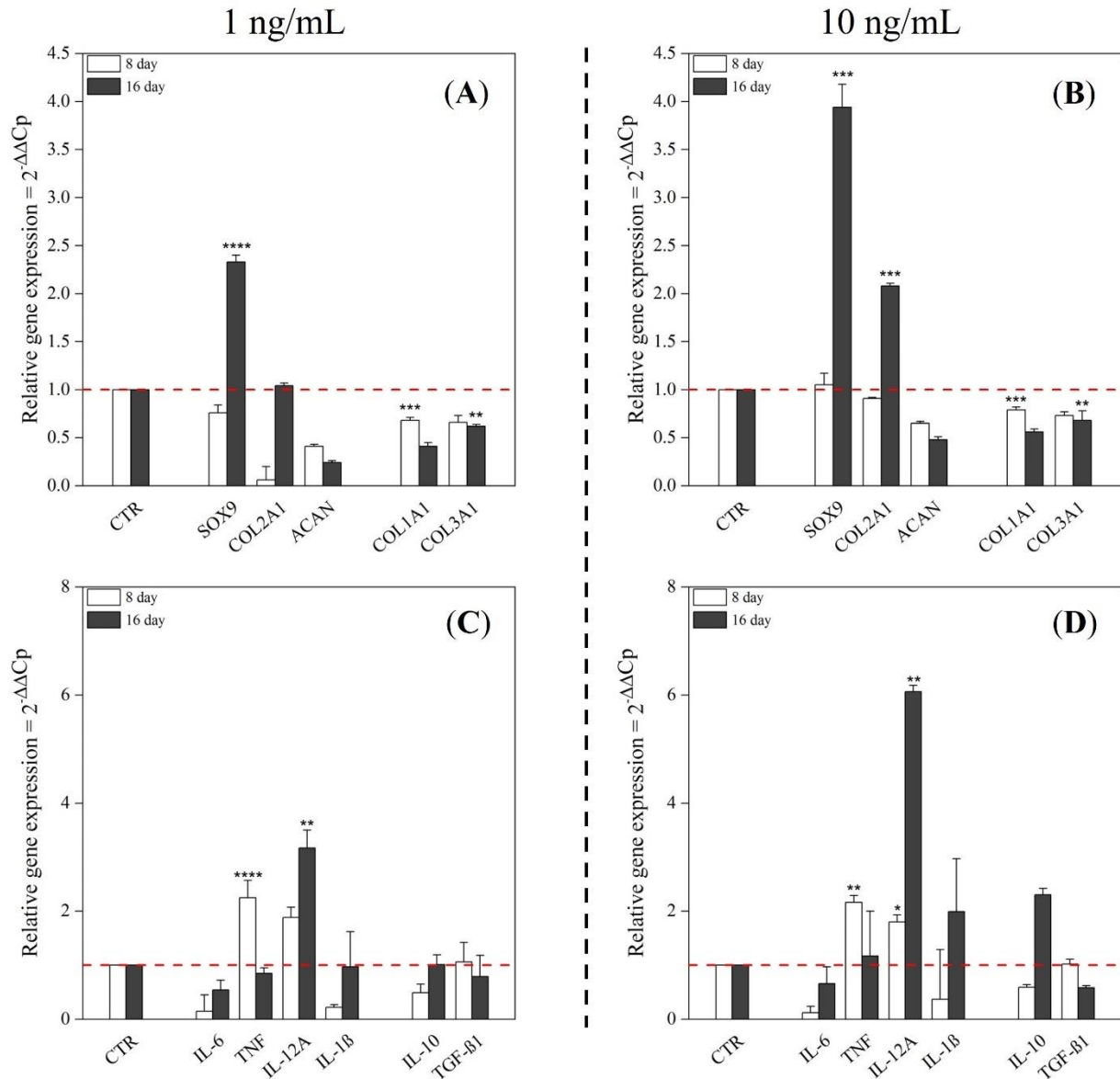


Figure 8 Effect of hTGF- β 1 on the expression of chondrogenic markers and cytokines by hBM-MSCs monolayer.

Two different concentrations of hTGF- β 1 (1 ng/mL and 10 ng/mL) for up to 16 days were tested. Untreated cells at matched time points were used as control. mRNA expression levels of positive and negative chondrogenic markers (*COL1A1*, *COL2A1*, *COL3A1*, *SOX9*, and *ACAN*) at 1 ng/mL (A) and at 10 ng/mL (B); pro- and anti-inflammatory cytokines (*IL-6*, *TNF- α* , *IL-12A*, *IL-1 β* , *IL-10*, and *TGF- β 1*) at 1 ng/mL (C) and at 10 ng/mL (D). All data were analyzed by two-way ANOVA, N=3 (biological replicates); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. control.

The effect of hTGF- β 1 on the production of type II and III collagen proteins was also monitored by semi-quantitative immunofluorescence (q-IF) assay (Figure 9A). hBM-MSCs displayed low basal expression of types II and III collagen that increased following 16 days of continuous hTGF- β 1

exposure; however, type II collagen staining (green) was more intense when cells were supplemented with 10 ng/mL of growth factor at Day 16. This behavior was confirmed by q-IF data, which indicated an increase (2.74-fold) of the stained protein signal at day 16 (**Figure 9B**). Supplementing 1 ng/mL, several spindle shaped aggregates were observed at day 16. No spindle systems were evidenced when a concentration of 10 ng/mL was used. Type III collagen signal was upregulated at Day 16 in all conditions when compared to cells at time zero, as confirmed by (**Figure 9C**), but, if it is compared with untreated cells at matched time-points, its intensity decreased following chondrogenic medium treatment.

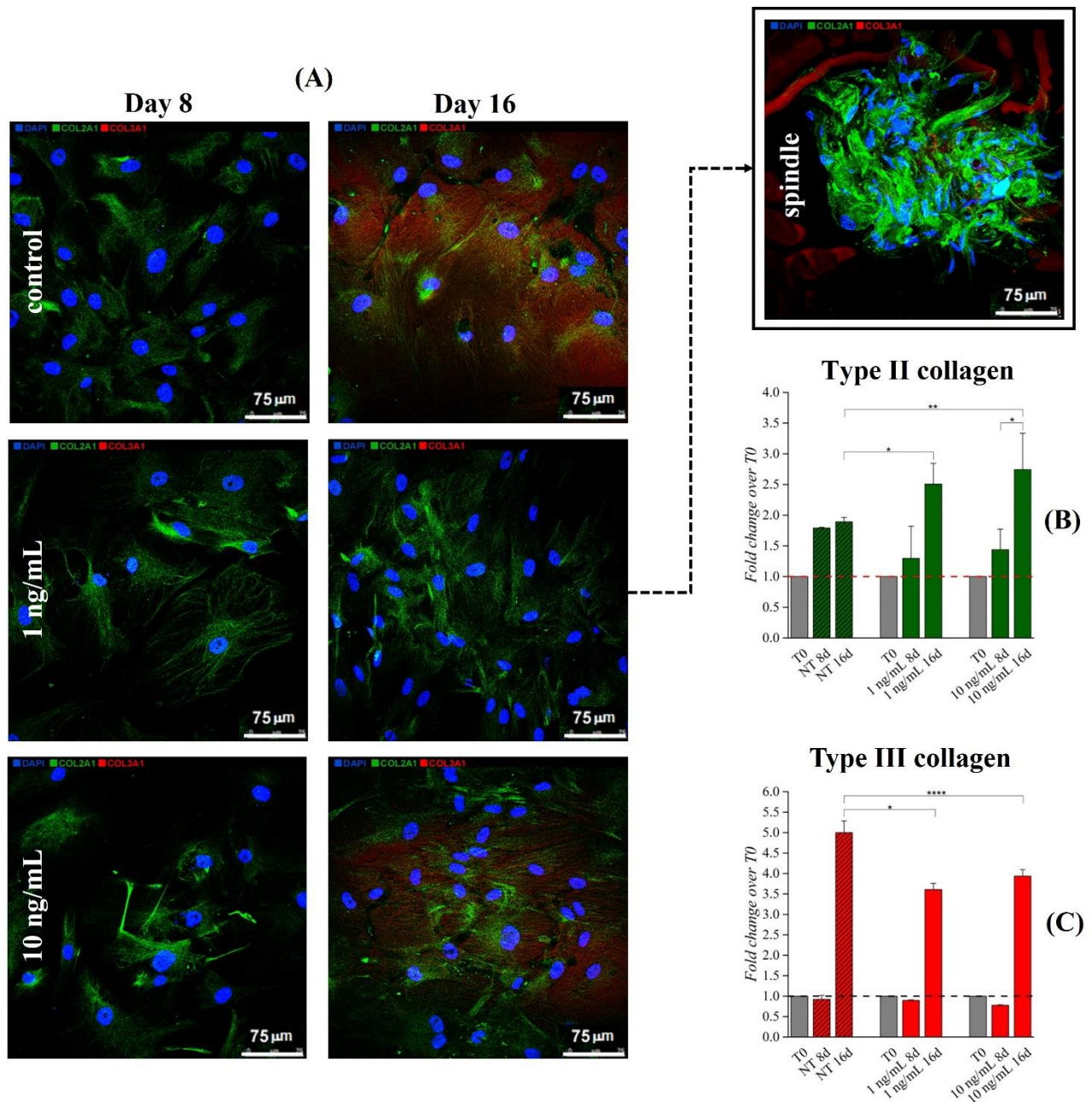


Figure 9 Effect of hTGF- β 1 on the expression of type II (stained in green) and type III (stained in red) collagens by BM-MSCs monolayer

Spindle-shaped systems were observed at Day 16 only when 1 ng/mL hTGF- β 1 was supplemented (A). Higher type I collagen stain was observed when 10 ng/mL were supplemented, as indicated by signals relative quantification (B, C) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, one-way ANOVA). N=2 (biological replicates). Scale bar: 75 μ m.

Based on these data, 10 ng/mL hTGF- β 1 was chosen as the optimal concentration for culture medium supplementation in the next 3D high-density approach.

2.4.2 Three-dimensional high-density culture

3D aggregates along 16 days of incubation were monitored using brightfield microscopy for morphological analysis. hBM-MSCs high density culture showed a spherical structure with smooth border, especially after 4 and 8 days (**Figure 10A**). A mean Feret's diameter of 450 μm (± 64) and circularity of 0.54 (± 0.16) were calculated after 16 days of treatment with hTGF- $\beta 1$; instead, control 3D culture without growth factor had, as previous ones, a mean diameter of about 460 μm (± 64), but a lower value of circularity roughly 0.46 (± 0.11). hWJ-MSC 3D systems displayed sizer and more regular aggregates, with a mean Feret's diameter of 1163 μm (± 69) and circularity of 0.62 (± 0.08) at the end of chondrogenic culture (**Figure 10B**).

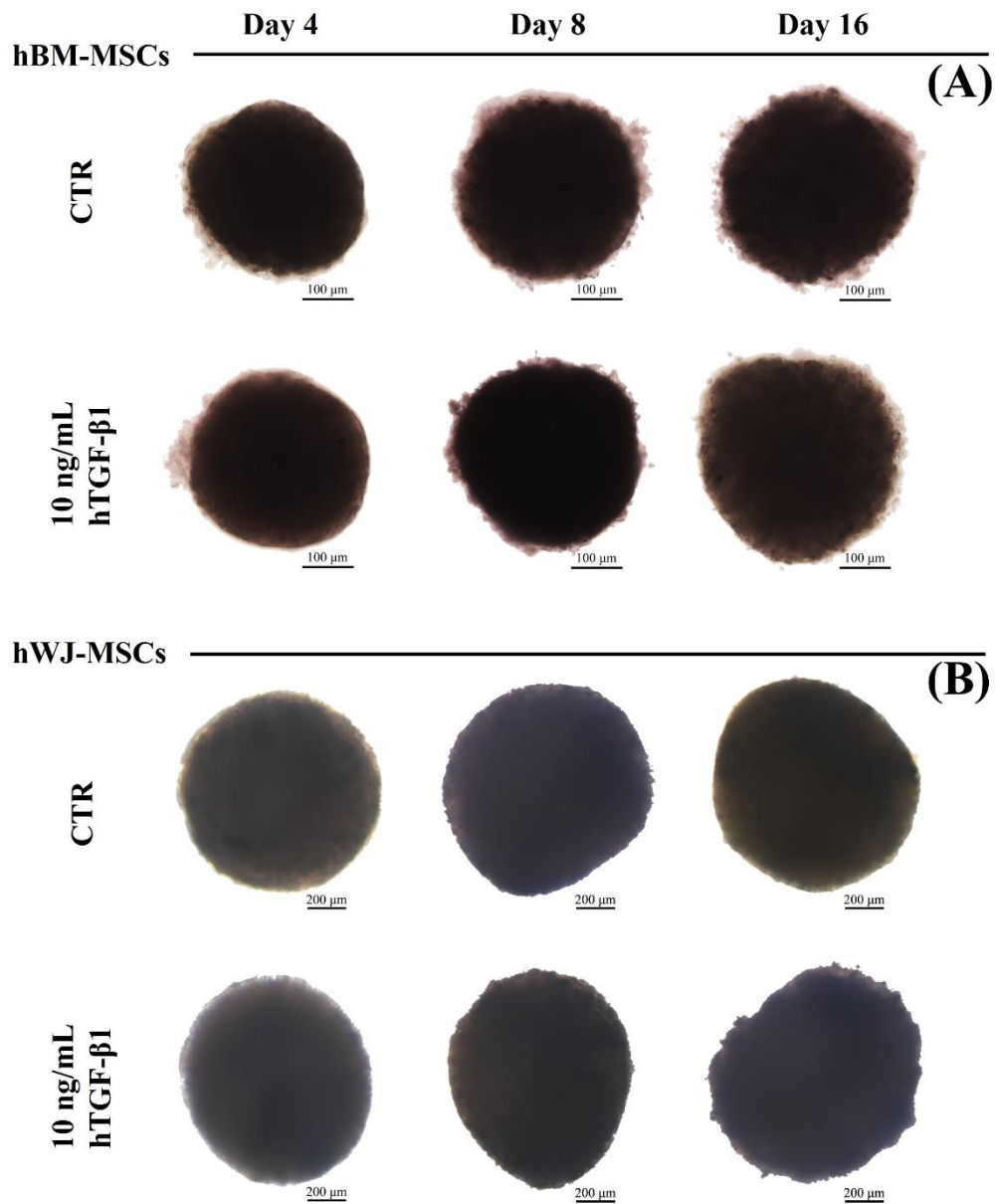


Figure 10 Brightfield images of 3D high-density cultures

hBM-MSCs **(A)** and hWJ-MSCs **(B)** 3D high-density cultures supplemented with 10 ng/mL of hTGF- β 1 and acquired at Days 4, 8, and 16. Untreated spheroids at matched time-points were presented for control purposes. Scale bar: up 100 μ m; down 200 μ m

Gene expression profiling of chondrogenic markers and cytokines were monitored along the experimental timeline. Specifically, all chondrogenic markers were upregulated in 3D culture combined with hTGF- β 1 treatment. At Day 8, hBM-MSCs within the 3D system displayed upregulation of *SOX9* (3.86-fold), *COL2A1* (353.3-fold), *ACAN* (0.89-fold), and *COL3A1* (2.40-fold). At Day 16, increased upregulation was noted for *SOX9* (12.99-fold, $p < 0.001$), *COL2A1* (1789-fold), and *ACAN* (30-fold), while *COL1A1* was downregulated and *COL3A1* unchanged with respect to control (**Figure 11A**). Despite the larger diameters observed, WJ-3D system at Day 8 displayed strong upregulation of *SOX9* (76.1-fold), *COL2A1* (75-fold), *COL1A1* (181-fold), and *COL3A1* (32.3-fold) while *ACAN* underwent modest downregulation. At Day 16, *SOX9* (222-fold, $p < 0.001$), *COL2A1* (390-fold), and *ACAN* (4.1-fold, $p < 0.05$) all underwent further upregulation, while *COL1A1* (67.6-fold, $p < 0.001$) and *COL3A1* (8.39-fold) displayed downregulation in comparison to day 8 levels (**Figure 11B**).

hBM-MSCs immunomodulatory activity resulted in transcriptional upregulation of all pro-inflammatory cytokines genes, although those most expressed were *TNF- α* and *IL-12A*, respectively 550-fold and 588-fold, at Day 8. On the other hand, among anti-inflammatory cytokines, *IL-10* showed a strong upregulation with a 3432-fold change. After 16 days, only *TNF- α* underwent a further increase (2120-fold, $p < 0.01$), whereas the other ones retained an expression similar to the previous time-point. Moreover, at the end of culture, *IL-10* was further upregulated (4255-fold, $p < 0.01$), instead, *TGF- β 1* retained a slight increase compared to control (**Figure 11C**). hWJ-MSCs Cytokine's expression resulted in limited transcriptional upregulation of all pro-inflammatory cytokines genes, with *IL-6* that reached the highest fold-value (15.4-fold) at Day 8; in contrast, *IL-10* showed a strong upregulation (77-fold). As culture progressed, *TNF- α* (54.57-fold, $p < 0.01$) and *IL-12A* (42.08-fold, $p < 0.001$) significantly increased their fold, while the other pro-inflammatory cytokines displayed a less shift. Moreover, *IL-10* and *TGF- β 1* expression increased compared to control, reaching respectively 608.87-fold ($p < 0.0001$) and 19.28-fold ($p < 0.05$) (**Figure 11D**).

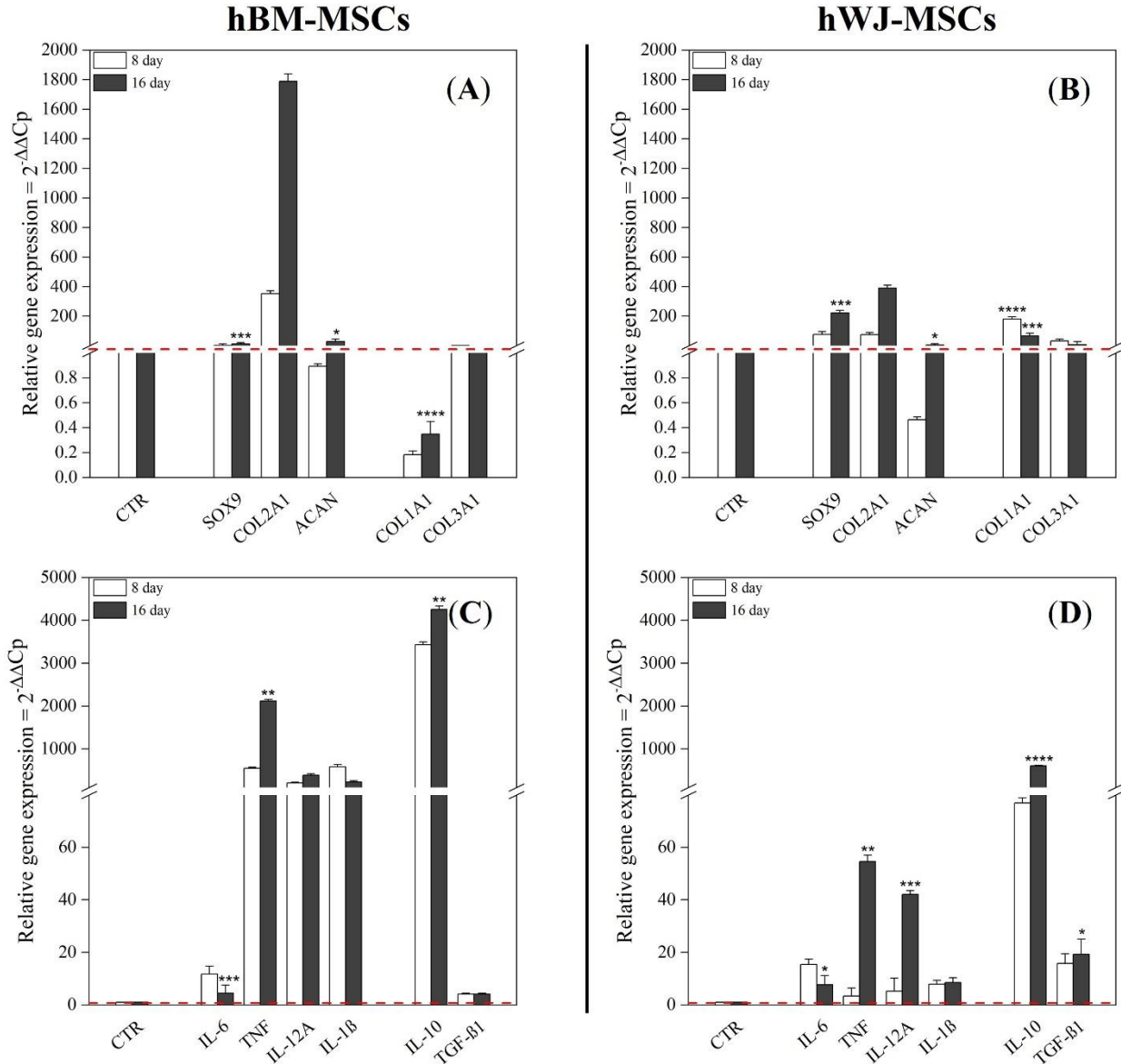


Figure 11 Gene expression profiles of both hBM-MSCs and hWJ-MSCs based high density culture supplemented with 10 ng/mL of hTGF-β1.

mRNA expression levels of positive and negative chondrogenic markers (*COL1A1*, *COL2A1*, *COL3A1*, *SOX9*, and *ACAN*) (A, B) and cytokines (*IL-6*, *TNF-α*, *IL-12A*, *IL-1β*, *IL-10*, and *TGF-β1*) (C, D); untreated cells at time zero were used as control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (two-way ANOVA). N=3 (Biological replicates).

Cells viability inside the 3D aggregate system was performed after 8 and 16 days of culture using Live and Dead assay, and findings related to BM and WJ spheroids are shown in (Figure 12A, 13A), where the red signal, associated with dead cells, was detectable only in the core of the 3D system and

varied from 5% at Day 7 to 20% at Day 16. Taking into account this data, cultures longer than 16 days were not considered for both cell type, as also suggested in literature ¹⁰¹.

Production of type I and II collagen (proteins) was also monitored by immunofluorescence IF assay (**Figure 12B, 13B**). WJ and BM-3D-systems displayed low expression of type I collagen, that decreased following 16 days of continuous hTGF- β 1 exposure; however, type II collagen staining (green) was more intense at Day 16. Histological evaluation by Sirius red, acquired with a polarized microscope to intercept the birefringence of collagen fibers, was performed. Intensity of the red signal, mainly associated with type II collagen, increased from 8 to 16 Day, confirming IF data. Translucent new fibers were also clearly detected at day 16 of culture (**Figure 14**).

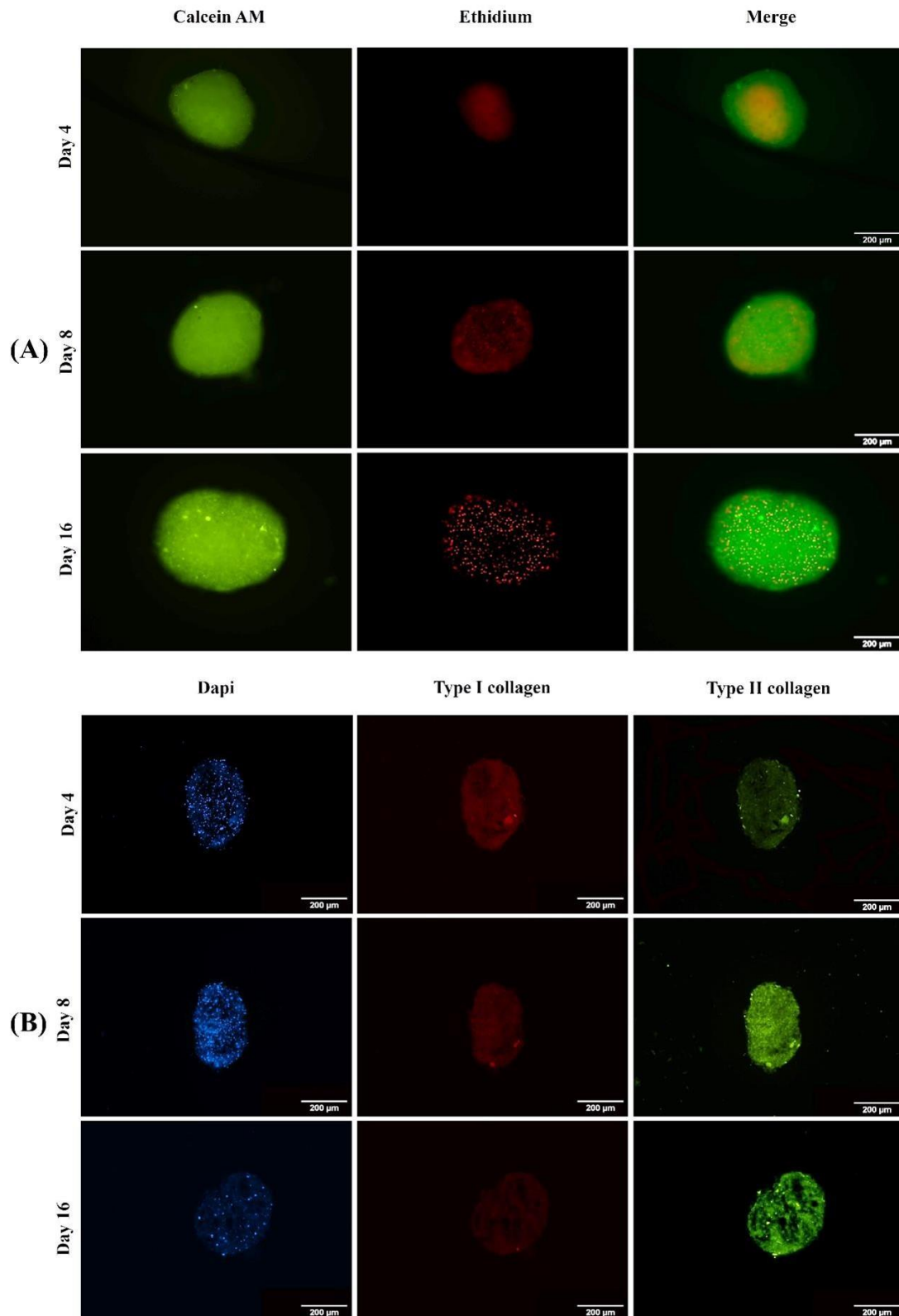


Figure 12 Live & Dead (A) and Immunofluorescence (B) of hBM-MSCs 3D high-density culture supplemented with 10 ng/mL of hTGF- β 1 and acquired at Days 4, 8, and 16.

Viable cells appear green, non-viable cells in red. Type I collagen was stained in red and type II collagen in green. DAPI was used to counterstain the nuclei (blue). Scale bar: 200 μm

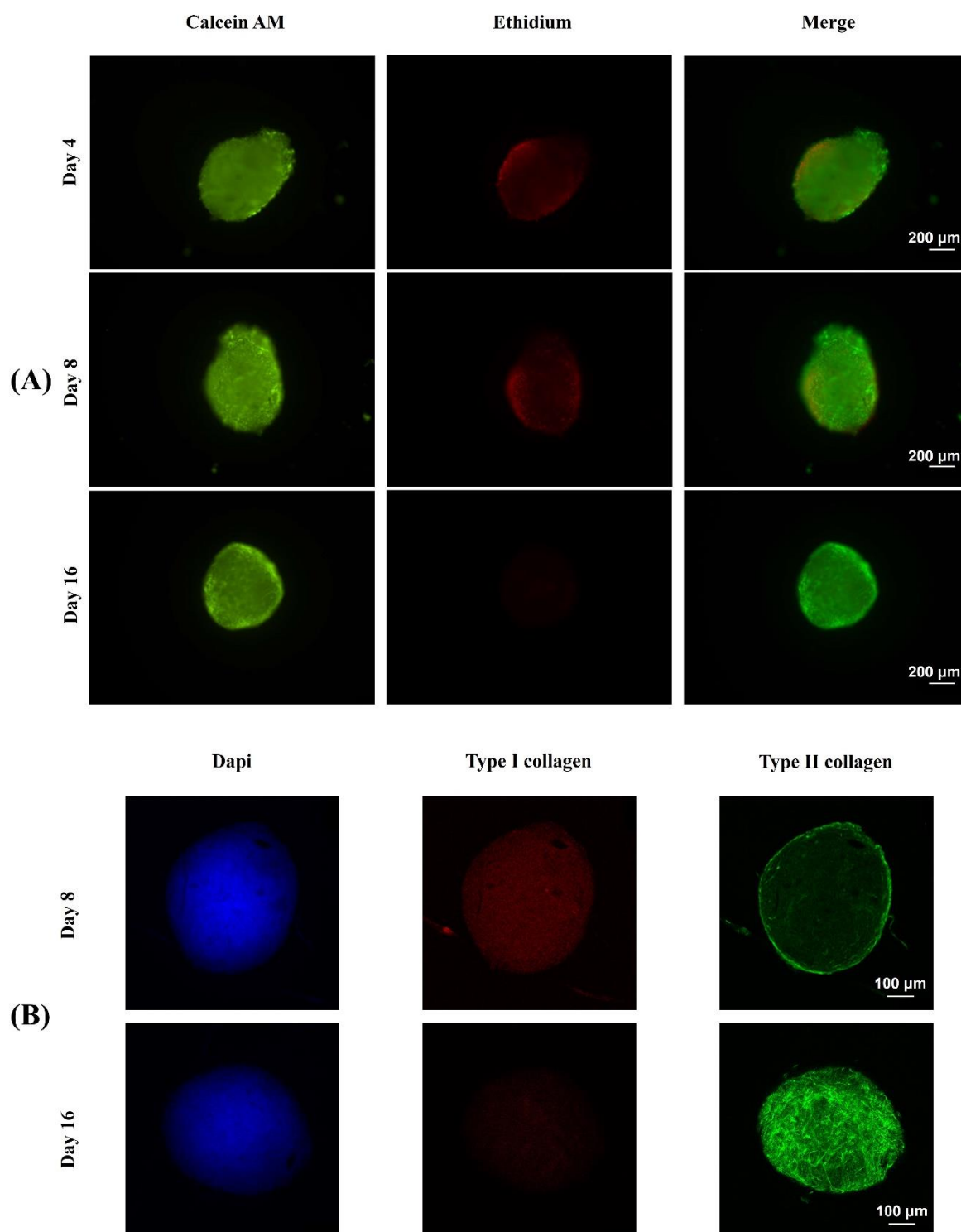


Figure 13 Live & Dead (A) and Immunofluorescence (B) of hWJ-MSCs 3D high-density culture supplemented with 10 ng/mL of hTGF-β1 and acquired at different time-point.

Viable cells appear green, non-viable cells in red. Type I collagen was stained in red and type II collagen in green. DAPI was used to counterstain the nuclei (blue). Scale bar: 200 μm

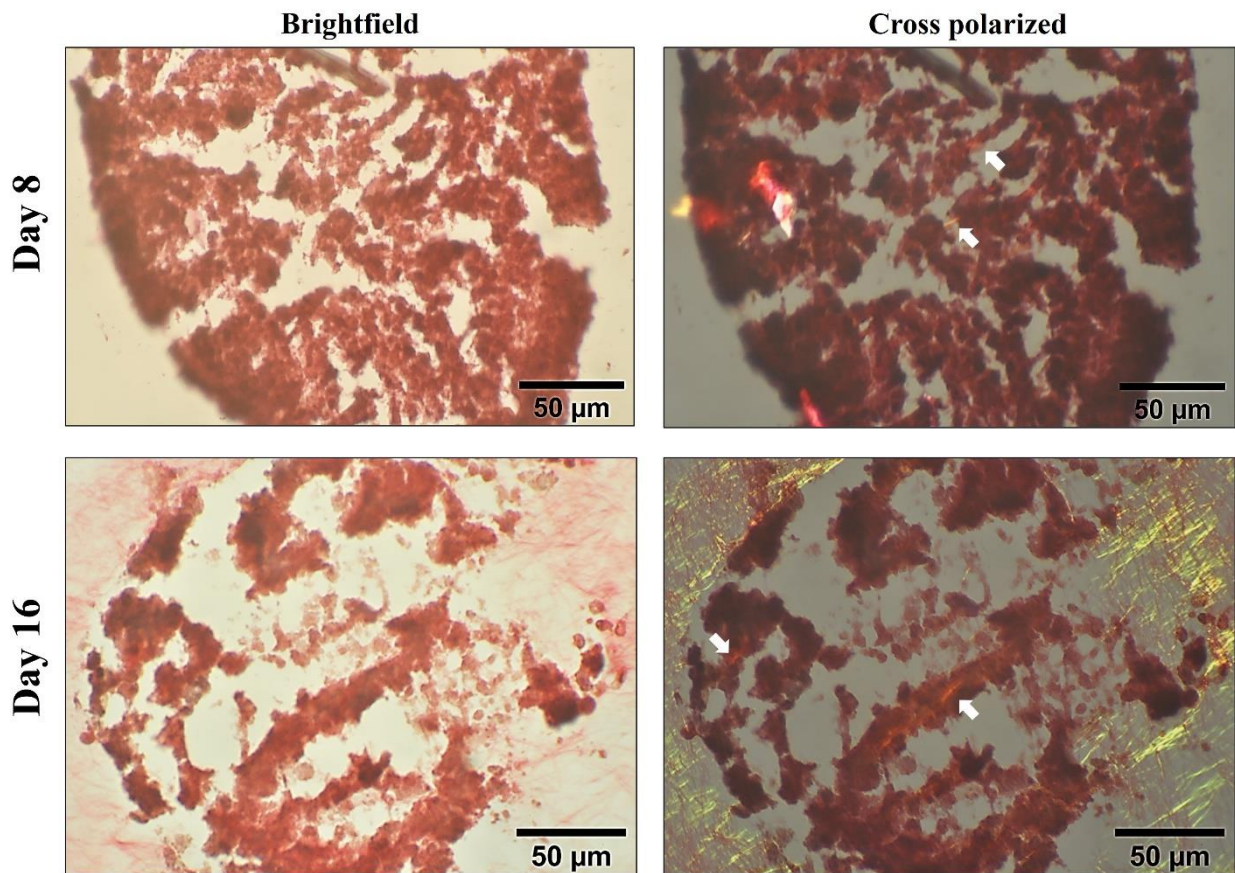


Figure 14 Brightfield and cross-polarized images of picosirius red stained hBM-MSCs based high density culture at days 8 and 16 (15μm thickness).

Fine birefringent collagen fibers were visible by day 8 and day 16 (see white arrows). At Day 16 birefringent fibers are visible, surrounding the sample. Scale bar: 50 μm.

2.5 Discussion

3D *in vitro* chondrogenic models using human stem cells derived from different tissues have great potential in cartilage healing and regeneration ⁴⁹. In this study, we have investigated chondrogenic propensity of human MSCs harvested from BM and WJ using 3D high-density culture coupled with hTGF-β1 delivery. We also studied variations in cytokine production during differentiation by gene expression profiling for chondrogenic commitment and pro- and anti-inflammatory cytokine genes.

Chondrogenesis from human MSCs is a complex process, involving the activation of different genes, especially the transcription factor *SOX9* that plays a key role in triggering the differentiation phase. Indeed, its upregulation promotes stem cell commitment towards chondrogenic phenotype and

reduces hypertrophic drift leading to cartilage mineralization, often accompanied by apoptosis of chondrocytes and matrix ossification^{102,103}. While *SOX9* is rapidly upregulated along chondrogenesis, the expression of other genes emerges later. *COL2A1* represents the gold standard among chondrogenic markers and is usually detectable only after several days of chondrogenic induction. In our monolayer experiments, chondrogenesis-related gene expression was not affected by 1 ng/mL hTGF- β 1 supplementation in culture medium, while increasing at 10 ng/mL after 16 days of culture, in agreement with previously published data¹⁰⁴. *ACAN*, another chondrogenic marker, was not detected at any time point at 1 and 10 ng/mL hTGF- β 1 concentrations, possibly because its expression becomes evident between 18-24 days of culture¹⁰⁵; however, some variations are described due to biological variabilities, number of passage used for culture, different culture media and TGF (β 1 or β 3) used, and cell type employed (e.g., adipose-, BM-, or WJ-derived MSCs)^{106–108}. Higher concentrations of hTGF- β 1 have already been reported to exert inhibitory effects on adipose-derived MSCs⁵⁶. Therefore, we did not test higher doses in our monolayer experiments for identification of optimal hTGF- β 1 concentrations for 3D culture systems while we chose 10 ng/mL concentration as the optimal condition because of significant increase in chondrogenic-related genes. Conversely, *COL1A1* and *COL3A1* were employed as negative markers because largely reported to be dedifferentiation genes of hyaline cartilage phenotype and often associated with differentiation towards other musculoskeletal lineages. Type I collagen gene expression is related with both tenogenic commitments (Ciardulli et al., 2020d) and fibrocartilage tissue (Kovermann et al., 2019). Moreover, its expression increases in chondrocytes during the progression of human osteoarthritis¹⁰⁹. Type III collagen is often co-localized with type I within the same fibril¹¹⁰, for this reason, it is also considered a negative marker too. However, its exact role in chondrogenesis remains unclear because contrasting evidence on its protective or pathological functions after injuries is reported¹¹¹. Development of a stellate or fibroblast-like phenotype during culture compromises the clinical outcome of potential regenerative therapy and is a major issue for the application of hBM-MSCs in

the repair of cartilage defects ¹¹². According to published literature, these genes were downregulated in response to 1 and 10 ng/mL hTGF- β 1 concentrations in our monolayer experiments confirming chondrogenic commitment of stem cells in our system culture. *In vitro* chondrogenic BM-MSC differentiation in 3D high-density cultures supplemented with TGF- β 1 or TGF- β 3 is often accompanied by up-regulation of genes associated with chondrocyte hypertrophy, including type X collagen and its transcription factor (RUNX-2), or matrix metalloproteinases (MMPs), and by activation of alkaline phosphatase (ALP). Hypertrophic processes are strongly dependent on type and concentrations of chondrogenic inducers used and also on *in vitro* culture time ¹¹³. In our study, the early phase of chondrogenic differentiation, also known as pre-differentiation or commitment phase, was investigated using a short culture period (only 16 days); therefore, hypertrophic processes should not have started in our 3D *in vitro* model; however, even though our system could not prevent hypertrophic drift of BM-derived MSCs, we showed a high chondrogenic potential of BM- and WJ-derived stem cells in 3D high-density conditions. WJ-MSCs are another possible source of mesenchymal stem cells for regenerative medicine approaches; however, few or absent data are present about hWJ-MSC chondrogenic commitment efficiency. In our study, we assumed that similar events to those occurring in hBM-MSCs may be induced in WJ cells. Similar to that reported in 2D BM-MSC culture with 10 ng/ml of TGF- β 1, WJ-MSCs showed *SOX9* upregulation at day 16, and *COL1A1* decreased expression from day 8 to 16. These results support the use of WJ-MSCs as a valid alternative for chondrogenic commitment of stem cells in regenerative medicine.

Monolayer culture systems have various limitations, especially in chondrogenic differentiation of human stem cells, because cells grow in adhesion on the flask bottom without mimicking the complex physiological *in vivo* tissue architecture. Therefore, when stem cells are cultured in 2D tend to lose their stemness and differentiation potential ¹¹⁴, as documented by the lack of *ACAN* expression due to the short culture period in order to avoid de-differentiation processes on long-term 2D chondrocyte cultures ¹¹⁵. Moreover, living 3D structures seem to be advantageous, especially in chondrogenesis

studies, due to the presence of a hypoxic core within a structure that would stimulate differentiation. Indeed, recent studies have confirmed that hypoxia is a full-fledged chondrogenic stimulating factor, because articular cartilage is an avascular tissue with a reduced oxygen and nutrient intake. Therefore, hypoxia seems to better reproduce the natural chondrocyte environment ^{116,117}. In our study, successfully 3D high-density cultures were obtained when 5×10^5 cells per mL were used. BM-MSCs 3D systems with a mean Feret's diameter of $401 \pm 39 \mu\text{m}$ and circularity of $0.60 \pm 0.14 \mu\text{m}$ were obtained after 4 days of culture with 10 ng/mL hTGF- β 1 supplemented medium. As culture moved forward, these systems became larger in size, but less regular in shape at Day 16, probably due to the proliferative effect of TGF- β 1 ¹¹⁸. Lower cell amounts resulted in smaller and frailer spheroids making extremely challenging further studies (data not shown). BM-3D system was more effective than 2D cultures in driving chondrogenesis, as confirmed by the higher upregulation of markers associated with hyaline cartilage, including *ACAN* at day 16, not observed in monolayer experiments. Moreover, *COL1A1* was significantly downregulated at day 16, confirming the capability of the 3D system to inhibit the expression of this gene ¹¹⁹. In contrast, hWJ-MSCs high-density culture showed smaller and separate spherical aggregate, with a mean Feret's diameter of $101 \pm 38 \mu\text{m}$ and circularity of 0.43 ± 0.12 at day 4 and reaching values of $104 \pm 42 \mu\text{m}$ and $0.36 \pm 0.07 \mu\text{m}$, respectively in diameter and circularity at the end of chondrogenic culture, thus showing a constant size trend but a slight decline in circularity over time. Different spheroid morphology between BM and WJ-MSCs could be caused by various patchy adhesion forces and a higher proliferative rate of WJ-MSCs compared to BM-MSCs. Indeed, spheroid formation can be influenced by different parameters, such as cell suspension density, manufacturing method, medium composition, or incubation time. Cells suspensions assemble into 3D pellets because of cell-to-matrix and cell-to-cell interactions via integrins and cadherins. Despite different size and morphology, WJ-spheroids overexpressed all chondrogenic markers, including *ACAN* at day 16 and *COL2A1*, with marked downregulation of

COL1A1 and *COL3A1* already starting from day 8 through day 16, suggesting that also WJ-3D system can be an effective *in vitro* tool for cartilage tissue engineering.

3D system also displayed good cell viability, even if small cell mortality was observed within the 3D system. This behavior could be due to hypoxic stress or low nutrient exchanges to which the cells within the system are subjected. Looking at immunofluorescence data of the 2D culture, up to 3-fold change of collagen II was measured by q-IF at day 16, coupled with a slight downregulation of collagen III along the culture (1-fold), especially when 10 ng were supplemented. A further observation must be done, when supplementing 1 ng/mL: i.e., in this condition a spindle-shaped aggregates were detected only at Day 16; these spindle structures may be considered an early organization of cellular 3D structure in the context of tenogenic commitment ¹²⁰. Therefore, it is suggested that the lower 1 ng/mL hTGF- β 1 dose may stimulate alternative differentiation pathways, while the higher concentration, 10 ng/mL hTGF- β 1, drives hBM-MSC chondrogenic commitment ¹²¹.

MSCs can have immunomodulatory activities as described in several hematological disorders, such as in primary myelofibrosis where they can also promote marrow fibrosis, or in acquired aplastic anemia where BM-MSCs maintain the physiological balance between T regulatory cells and T helper(Th) 17 lymphocytes in the BM niche ¹²². Moreover, BM-MSCs show upregulation of Th1, Th2, and Th17 differentiation-related and inflammation-associated genes in aplastic anemia. However, modifications of cytokine expression during chondrogenic commitment in both 2D and 3D culture systems using both BM- and WJ-derived MSCs have not been investigated yet. In our study, we demonstrated that when supplementing 10 ng/mL of hTGF- β 1 in 2D system, TNF- α and IL-12A, two pro-inflammatory cytokines, were upregulated starting from day 8 of culture. TNF- α and IL-1 β might hamper chondrogenesis of mesenchymal stromal cells, through NF- κ B pathway, inhibition of SOX9 expression and increased synthesis of degradative enzymes involved in cartilage degeneration,

like matrix metalloproteinases (MMPs) ¹²³. However, other studies suggest that *in vitro* exposure to TNF- α might increase proliferation, migration, and osteogenic differentiation of MSCs ¹²⁴. Our results from cytokine expression combined with chondrogenic-related genes support the hypothesis that autocrine TNF- α production by MSCs might promote chondrogenesis in *in vitro* models. This effect was even more pronounced in 3D systems. Indeed, BM-3D culture showed an upregulation of all pro-inflammatory cytokine genes explored at Day 8, except for *IL-6* that showed a poor upregulation followed by a decrease at Day 16. The behavior of this particular cytokine is in agreement with a previous study, where *IL-6* expression, during chondrogenic differentiation by pellet culture, decreased from Day 8 to Day 16 ¹²⁵. During the chondrogenic commitment, the cytokine most expressed was *IL-10*, as we also have observed, suggesting its involvement in driving differentiation ¹²⁶. Similarly, in 3D-WJ culture, cytokine expression profile was characterized by a similar upregulation trend of both *IL-6* and *IL-10* compared to BM-3D system. Therefore, both BM- and WJ-derived MSCs can produce pro-inflammatory cytokines that might support the chondrogenic process in an autocrine manner, as proposed by a more pronounced variations in chondrogenic-related genes in 3D systems. Indeed, cell-to-cell contacts and spheroid structure in 3D culture resemble the physiological architecture of tissues and reproduce interactions between cells and ECM.

2.6 Conclusion

In vitro models to investigate cartilage regeneration process are becoming increasingly important. Our results indicate that 3D high-density stem cell culture supplemented with 10 ng/mL of hTGF- β 1, represent a simple and rapid 3D model of chondrogenic commitment. These 3D biomaterials are extremely versatile tools and could be used to evaluate the effect of several drugs and growth factors aimed at cartilage regeneration and healing, as well as to explore the role of cytokines in leading chondrogenic commitment. Indeed, 3D-culture systems highlighted a well-balanced expression of

pro and anti-inflammatory cytokines, especially of *IL-10* at Day 16, suggesting their involvement in chondrogenic events. This trend was observed for both BM- and WJ-3D systems and provided an important correlation between chondrogenic commitment that might be sustained by an autocrine cytokine production. The described data open also interesting prospects on the use of hWJ-MSCs to develop 3D models for cartilage tissue engineering.

CHAPTER 3: Microencapsulation of TGF- β 1:
the new frontier of tissue engineering

3.1 Introduction

Micro and nano-carriers, with controlled size and suitable encapsulation efficiency, may act as micro-environmental regulators within a 3D bioengineered scaffold, providing delivery of a wide range of spatio-temporally controlled bio-molecule delivery^{127,128}. These 3D assembled synthetic matrices can be used for highly predictive *in vitro* models or to fabricate implantable devices for tissue engineering applications^{129–132}. In this context, controlled release systems have recently received extensive attention given their great potential not only in pharmaceutical^{133–135} but also in biomedical applications within tissue engineering protocols^{136–138}. Particularly, growth factors are small biomolecules capable of stimulating cellular growth, proliferation, and differentiation¹³⁹. Therefore, they are largely used in tissue engineering protocols to induce stem cell commitment towards specific phenotype(s). For example, human GDF-5 induces the expression of genes typically associated with the tendon phenotype^{131,140} and for this reason, it is used in pharmacological protocols for tendon healing and repair. On the contrary, hTGF- β 1 has been widely investigated in the process of chondrogenesis for cartilage healing protocols^{141,142}.

PLGA and PLA are FDA-approved bioresorbable carriers^{143,144} capable of bulk degradation without swelling and with a recognized capacity of ensuring controlled release rate of loaded drugs^{138,145–148}. Several techniques are available to prepare biopolymer-based micro/nano-carriers, such as spray drying^{149,150} or coacervation¹⁵¹, even though, in the specific case of PLA and PLGA processing, the solvent evaporation/extraction of emulsions. This has been described as optimal given its milder processing conditions compared to spray drying, and easier solvent removal compared to coacervation. Indeed, during the evaporation or extraction of emulsion, the oily droplets dispersed in the water external phase are hardened leading to micro/nano-carriers formation^{152,153}. Despite these two techniques being largely recommended, evaporation showed batch to batch reproducibility disturbances^{154,155} and extraction uses relatively large amounts of a second solvent producing the problem of solvent mixtures recovery. Both processes require a processing time of several hours and,

as a consequence, aggregation phenomena may occur between the droplets, producing carriers with a larger poly-dispersity compared to derived droplets ¹⁵⁶. Another disadvantage lies in the poor encapsulation efficiencies of high water-soluble compounds ¹⁵². Indeed, these molecules could spread out or diffuse from the dispersed oily phase into the aqueous continuous phase, and their microcrystalline fragments could deposit on the particulate surface and/or outside of the biopolymer matrix ¹⁵². It should also be underlined that despite the widespread use of solvent evaporation/extraction processes to prepare polymer-based carriers, there are no standard protocols acquired, and each preparation follows its own set.

Dense carbon dioxide technologies have been proposed to produce micro/nano-particles or biopolymer 3D scaffolds for tissue engineering applications ¹²⁸. SEE technology operating in a continuous layout using a counter-current packed tower ¹⁵⁷ was described for PLA/PLGA carriers fabrication. SEE ensured improved performances, such as better batch-to-batch reproducibility ¹⁵⁸, accurate carrier size control thanks to a fixed droplet shrinkage without aggregation phenomena ¹⁵⁹, lower solvent residue ¹⁶⁰ and controlled encapsulation efficiency ^{161–163}.

In contrast to SEE other supercritical technologies described for particle formation cannot properly work in the case of PLA/PLGA carriers preparation for different reasons: for example, impregnation technology with supercritical fluid cannot be properly applied to PLGA and PLA carriers fabrication because of well-documented polymer foaming ¹⁶⁴. Furthermore, considering PGSS or Supercritical Assisted Atomization ^{165,166} technologies, the supercritical or dense carbon dioxide comes in direct contact with the polymer solution before the expansion step and the subsequent particles' precipitation. Particles are then recovered in a dried environment that cannot be applied to PLA/PLGA. Indeed, these biopolymers when in contact with dense-CO₂, undergo a reduction of their glass transition temperature that becomes close to the processing temperature of 311-312 K, with consequent particle aggregation or film formation. This behavior prevented the processing of these

biopolymers by SAS ^{167–169}; indeed, when PLA/PLGA micronization is attempted by SAS, larger particle aggregates or a continuous film have been always collected (personal communication, data not published).

Furthermore, PGSS, SAA, and SAS technologies are not suitable for peptide processing, because of the use of organic solvents to solubilize polymer and peptides which induces their denaturation. This adverse condition is not present when using SEE technology. Indeed, carriers are collected in a liquid environment (water external phase) that prevents the aggregation of any particles and the growth factors are well preserved within the internal water phase before droplet drying ^{135,137}. Moreover, given the Joule-Thomson effect consequent to the expansion of carbon dioxide, the water suspension is always maintained refrigerated in the collection vessel.

However, despite the large investigation on SEE carriers' formulation in terms of size distributions ¹⁷⁰ and drug loading optimization ¹⁶², to date, few investigations described the cytotoxicity of the produced particles. Particularly, *in vitro* cytotoxicity tests, including cell viability and proliferation studies are considered important screening assays, capable of evaluating the reaction of living cells to the implant and assessing safety characteristics of the produced carriers. Up to now, very few cytotoxicity characterizations have been described for the micro/nano materials fabricated by dense gases, while several assays have been performed for carrier fabrication using conventional technologies ^{171,172}. Different cell models can be used to assess the cytotoxic activity of biomaterials. For example, Chinese Hamster Ovary cells (CHO-K1) are mitotic primary cells and, therefore, may provide indications on genotoxicity ¹⁷³. Human-Peripheral Blood Mononuclear Cells (hPBMCs), isolated from human healthy donors can provide indications of the immune reaction to the biopolymer devices and can better mimic *in vivo* conditions ^{174,175}.

3.2 Aims

Several emulsion formulation protocols with different surfactant amounts were tested and then, processed by SEE using both PLA and PLGA with different molecular weights or co-polymer ratios to fix carrier size and distribution. Then, human recombinant growth factor (hTGF- β 1) was added to the formulation to provide data about the drug loading and related release profiles. Cytotoxicity was monitored using two different cell types, from animal and human sources, namely CHO-K1 and hPBMCs, and a comparison was made with carriers obtained by conventional solvent evaporation. These data are extremely important for carriers' use in *in vitro* and *in vivo* protocols and to provide a better characterization of the safety of the SEE fabricated carriers.

3.3 Materials and Methods

3.3.1 Materials

Poly-Lactic-Acid (PLA, RESOMER 203H (Mw 18,000-24,000 Da, indicated as low Mw-PLA), Evonik Industries, Essen, DE) and Poly-lactic-co-Glycolic-Acid (RESOMER 504H, ratio 50:50 Mw 38,000-54,000 Da, indicated as high Mw-PLGA and RESOMER 752H, ratio 75:25, Mw 4,000-15,000 Da, indicated as low Mw-PLGA, Evonik Industries, Essen, DE) were used as received. O₂ (99.9%, Morlando Group, Naples, IT).

Polyvinyl alcohol (PVA, Mol wt: 30,000–55,000, Aldrich Chemical Co., Milan, IT), Bovine Serum Albumin (Sigma-Aldrich, Milan, IT), human Serum Albumin ($\geq$ 98%, HSA, Aldrich Chemical Co., Milan, IT), Tween 80 (Sigma-Aldrich, Milan, IT), Ethyl Acetate (EA, 99.9%, Aldrich Chemical Co., Milan, IT), recombinant hTGF- β 1 (PeproTech; UK), glucose (Sigma-Aldrich, Milan, IT), PBS 1X (Corning Cellgro, Manassas, VA, USA), Minimum Essential Medium Alpha (α -MEM) (Corning Cellgro, Manassas, VA, USA), Enzyme Linked Immunosorbent Assay (ELISA, Cloud-Clone Corp., USA), Ficoll-Hypaque gradient density (Sigma-Aldrich, Milan, IT), Roswell Park Memorial Institute

(RPMI, Life Technologies Inc., USA), fetal bovine serum (FBS) (Life Technologies Inc.), L-glutamine (Life Technologies Inc., USA), penicillin/streptomycin (Life Technologies Inc., USA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, Milan, IT), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, IT).

3.3.2 Emulsion preparation

Emulsions were obtained using a ratio of 1:19:80 *water-oil-water* ($w_1/o/w_2$). Oily phase was prepared by solubilizing a given amount of polymer, whereas a variable amount of PBS solution (1X) containing HSA (1% w/v) and PVA (0.06% w/w) was used as water internal phase. Recombinant hTGF- β 1 was reconstituted in citric acid (pH 3) to a final concentration of 1 $\mu\text{g}/\mu\text{L}$. w_1 was mixed with the oily phase to form a primary w_1/o emulsion by sonication with a digital ultrasonic probe operating at 30% of its amplitude (mod. S-450D; Branson Ultrasonic Corp. Danbury, Connecticut, USA). When growth factor was added, the inner water phase was mixed with the oily phase by vortexing (mod. Velp Scientifica, Monza, IT) at maximum speed for 30 sec.

The primary emulsion was then slowly added into EA-saturated aqueous Tween80 solution by high-speed stirring (mod. L4RT, Silverson Machines Ltd., Waterside, Chesham, Bucks, UK). Surfactant amounts in the external water phase varied from 0.1 to 0.6% w/w. In some cases, 15% w/w of glucose was added in the water external phase. Temperature of 10°C was assured during emulsion phase mixing. All emulsions were processed by SEE immediately after their preparation.

3.3.3 Dense gas technology protocol

SEE is a continuous process that involves the continuous counter-current extraction of solvent in the oily phase of emulsion by supercritical carbon dioxide. The prototype apparatus consists of a packed

column with stainless steel pickings (1889 m^{-1} specific surface and 0.94 voidage; Pro-Pak, Scientific Development Company, State College, PA, USA) where gaseous and liquid phases are in contact counter-current. The main apparatus system is formed by three extraction cascades in which the emulsion is introduced at the top, while CO_2 from the bottom, so counter-current flows are obtained¹⁵⁷. The three stages are formed by AISI 316 stainless steel cylindrical sections of 30 cm height, connected by four cross-unions. A refrigerated cyclonic separator is located downstream the top of the column for oily phase recovery through a micrometric valve. Another separator is located at the bottom of the column to collect the water external phase with carriers. The high-pressure diaphragm pump (mod. Milroyal B, Milton Roy, Pont Saint-Pierre, FR) is used to deliver the dense- CO_2 at a selected flow rate; a high pressure piston pump (mod. 305, Gilson, FR) is used to feed the emulsion at a constant flow rate. A rotameter and a dry test meter, located at the exit of the separator, measure the CO_2 flow rate and the total amount delivered, respectively. The column is thermally isolated by ceramic cloths, and temperatures profiles are controlled by six controllers, located at different heights of column¹⁷⁶. The SEE process scheme with a continuous counter-current operation is shown in **Figure 15c**.

To optimize the performance, it is necessary to reach a steady-state condition in the apparatus; proper gas/liquid mixing by complete wetting of the packing element of the column. This pre-conditioning operation was performed using a *w-o-w* emulsion formed by only solvents and surfactants. Finally, a washing step is performed to recover the carriers still retained in the column packing elements. Normally, 70% of overall polymer used in the emulsion is recovered for each run as a result of the loss of emulsion/suspension caused by dead volumes of SEE apparatus. The organic solvent recovered is less than 50% in weight of the solvent loaded in the emulsion, due to partial loss in the gas stream at the exit of the separator located at the top of the column.

PLA and PLGA carriers are not amenable to conventional sterilization methods such as ethanol washing, gamma irradiation, or steam because of biopolymer oxidation¹⁷⁷ and further degradation of peptide payload. To overcome these challenges, a specific SEE operational protocol was set. Carriers, collected from each run, were always washed in sterile conditions with a penicillin/streptomycin (1% w/v) and amphotericin (1% w/v) solution to ensure removal of the surfactant and to ensure cell culture-grade preparation, centrifuged for 50 min at 6500 rpm at 4° C (model IEC CL30R Thermo Scientific, Rodano, Milan, IT), recovered by using membrane filtration (filter 0.2 µm HA Millipore, Sigma Aldrich, Milan, IT). All experiments were repeated twice (N=2).

3.3.4 Solvent evaporation protocol

SE was performed to compare the outcomes of conventional and supercritical techniques starting from the same emulsion. In the case of SE, emulsions were stirred at 100 rpm for 3 hours at a temperature of 38°C in a sterile controlled environment, to allow solvent elimination by evaporation. Carriers collected from each assay were washed and centrifuged following the same protocol described above after for SEE process.

3.3.5 Morphological and size distribution analyses

The droplets of the emulsions were observed using an optical microscope (mod. BX 50 Olympus, Tokyo, JP) equipped with a phase contrast condenser. Droplet size distributions (DSDs) and Particle Size distributions (PSDs) were measured by dynamic light scattering (DLS, mod. Mastersizer S, Malvern Instruments Ltd., Worcestershire, UK). Analyses were performed using several milligrams of each sample (corresponding to more than one million droplets or particles). Size evaluation analyses were performed in triplicate. Mean as well as standard deviation were calculated for all data. The shrinkage factor percentage (SF%) was calculated as indicated in equation 1:

$$\text{Shrinkage (\%)} = \left(1 - \frac{\text{carriers mean size}}{\text{droplets mean size}}\right) \times 100 \quad (\text{eq.1})$$

Carrier morphology was observed using a Field Emission-Scanning Electron Microscope (FE-SEM mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, DE). Powder samples of each test were placed on a double-sided adhesive carbon tape previously glued to an aluminum stub and coated with a gold film (250 Å thickness) using a sputter coater (mod.108 Å; Agar Scientific, Stansted, UK).

3.3.6 hTGF-β1 release study and encapsulation efficiency

Growth factor release profile was monitored *in vitro* by suspending approximately 5 (±0.3) mg of carriers in 0.5mL of Phosphate buffered saline (PBS) plus 0.1% w/w Tween 20, which was placed in an incubator at 37°C and stirred continuously at 100 rpm. Every 24 hours, the samples were centrifuged at 12000 rcf for 10 min and the supernatant was completely removed and replaced with fresh media to maintain sink conditions. The concentration of the released peptides was then measured with an Enzyme Linked Immunosorbent Assay (ELISA, Cloud-Clone Corp., USA). Release experiments were performed in duplicate (N=2) and the curve describes the mean profile was calculated as ng of growth factor released from 100mg of polymer versus time. Encapsulation Efficiency (%) was defined as the ratio between loaded peptides in the emulsion and recovered per g of polymer; this value is expressed as a percentage.

3.3.7 Cell isolation and harvesting

(CHO-K1, ATCC® CCL-61™) were seeded in 96-well plates at a density of 10.000 cells/well and cultured in HAM's F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin (50 U/mL). Cells were then incubated in a humidified atmosphere containing 5% CO₂ and 95% air.

hPBMCs were obtained from three healthy donors (age 25-40 years) and separated by Ficoll-Hypaque gradient density (Sigma-Aldrich) following standard techniques ¹⁷⁴. All donors gave their written informed consent in accordance with the Declaration of Helsinki for the use of their residual blood for research purposes, with approval from the University Hospital of Salerno Institutional Review Board. After isolation, cells were re-suspended in RPMI medium, supplemented with 10% heat-inactivated FBS, 10 mg/mL L-glutamine, and penicillin/streptomycin (50 U/mL). Cells were then seeded in 96-well plates at a density of 10.000 cells/well and incubated in a humidified atmosphere containing 5% CO₂ and 95% air.

3.3.8 Cytotoxic activity

Cell metabolic activity was analyzed using the MTT assay. Briefly, cells were treated with decreasing amounts of growth factor loaded and unloaded PLGA and PLA carriers; pure growth factors were also tested at different concentrations for 24 and 48h. All carriers were suspended in culture medium using ultrasound in ice-cold (Elmasonic P, Elma Schmidbauer GmbH, DE). Following treatment, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added (1 mg/mL) to each well and incubated at 37°C for an additional 4 h. Plates were centrifuged at 300 g for 10 min and the supernatants completely removed. Formazan products were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was determined at 490 nm using a microplate reader (Infinite F200 PRO, Tecan Group Ltd., SW). All assays were performed in triplicate (N=3). For the MTT assay on hPBMCs, three independent experiments were performed in triplicate, each one on a single subject (N=3). Cell metabolic activity, a surrogate of viability, was calculated as the percentage of the control group, considered as 100%. The percentage viability of cells was calculated according to equation (2):

$$\text{Cell viability (\%)} = \frac{\text{Abs of sample} - \text{Abs blank}}{\text{Abs of control} - \text{Abs blank}} \times 100 \quad (\text{eq.2}).$$

3.4 Results and discussion

3.4.1 SEE operative conditions

SEE pressure and temperature operating conditions were selected to extract the solvent from the oily phase, as illustrated in the schematic representation (**Figure 15a**). Taking into account the high-pressure vapor-liquid equilibrium of the pseudo-binary system EA/CO₂, pressure and temperature conditions were chosen at 8 MPa and 312K to selectively extract the organic solvent from the oily phase of emulsion (see **Figure 15b**), adapted from ¹⁷⁸. Adopting this operative pressure and temperature, the approximate operative point (in the steady state operation) is indicated as the blue dot in **Figure 15b** and it is located outside the miscibility hole of the system EA plus CO₂. Therefore, the complete miscibility of the ethyl acetate in dense carbon dioxide was assumed. The approximated operative point position is also defined by Liquid/Gas flow rate ratio fixed at 0.1 (with CO₂ flow rate of 1.4 kg/h), according to the previous optimization ^{157,162,179}. In these condition, the water suspension recovery displayed a solvent residue less than 100 ppm ¹⁷⁶.

In these conditions, the external water phase of emulsion showed a negligible solubility in dense-CO₂ ¹⁸⁰ even when water phase pH was lowered to 5.2; this value was taken as acceptable for PLA/PLGA carrier production. Indeed, even if biopolymer degradation is promoted in aqueous medium, it is strongly inhibited by acid pH ¹⁴⁵; therefore, the presence of carbonic acid in the water medium would be also useful to ensure the chemical stability of biopolymers. Operating at 8 MPa and 311 K, CO₂ density is 304 kg/m³ and water density is 993 kg/m³. These conditions assured sufficient difference in density to enable counter-current flows ^{181,182}.

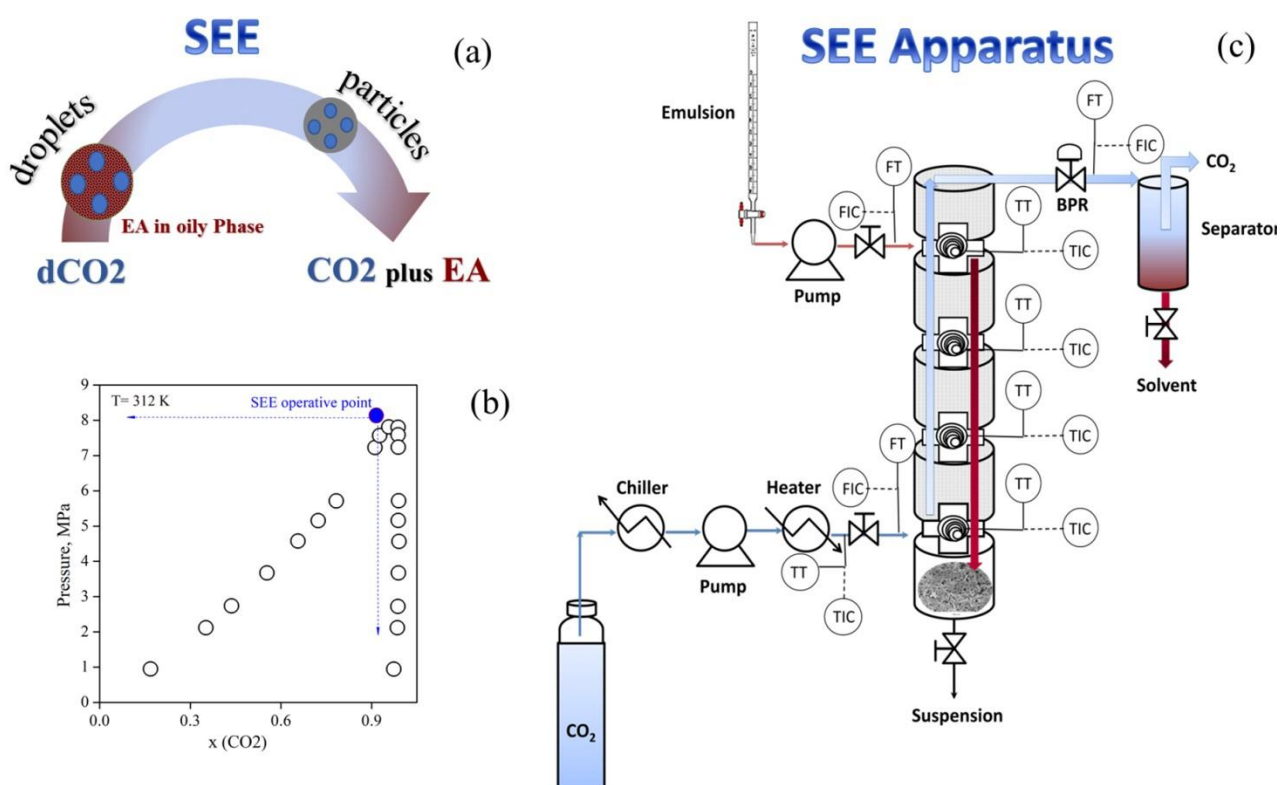


Figure 15 Schematic illustration of SEE process and apparatus

Schematic illustration of SEE process with the representation of droplet (in emulsion) shrinkage and polymer particle formation after the oily phase extraction by dense-CO₂ (a). Vapour Liquid Equilibrium (VLE) at high pressure for the system EA-CO₂ at 312 K adapted by Chrisochou et al., (1995); the blue dot approximatively indicates the operative point, neglecting the water phase that is immiscible in CO₂ in the same conditions (b). SEE apparatus layout with the packed column operating in counter-current mode; other legends: CO₂ supply; chiller used for CO₂ cooling; diaphragm pump used for high pressure CO₂; heater used for SC-CO₂ heating; emulsion supply; piston pump used for the emulsion; TT-TIC, temperature control; FT-FIC, flow rate control; BPR, back pressure regulator; separator used for CO₂ and solvent recovery; suspension recovery (c).

3.4.2 Emulsion Formulation Optimization

The surfactant of the external water phase was varied to optimize carrier size and distribution. The extremely low amount of growth factor used in the internal water phase was assumed not to exert any influence on carriers' morphology and size. A growth factor was loaded in the emulsion formulations previously optimized, to observe their encapsulation efficiency and release profiles as well as cytotoxicity. A summary of all tested compositions and results in terms of mean diameter, polydispersity, and loading are summarized in Error. L'origine riferimento non è stata trovata..

Table 3 A summary of emulsion formulations processed with SEE technology.

Internal water phase was always added with human Serum Albumin (1 % w/w); the proteins was used as growth factors stabilizer.

Polymer	Tween80 % w/w	Emulsion composition	DSD (±SD) nm	PSD (±SD) nm	SF %	Load µg/g	EE %
low Mw-PLA	0.1%	w1: 0.5mL water plus 0.06% PVA	1792±254	634±88	65	--	--
	0.3%	o: 1 g PLA in 19.5 mL EA. w2: 80 mL of EA _{sat} -W + Tween80	1613±335	285±45	82	--	--
	0.6%		819±110	220±41	73	--	--
low Mw-PLGA, 75:25	0.1%	w1: 0.5mL water plus 0.06% PVA	5554±555	342±90	94	--	--
	0.3%	o: 1 g PLGA in 19.5 mL EA. w2: 80 mL of EA _{sat} -W plus Tween80	2417±283	261±39	89	--	--
	0.6%		1388±259	205±32	85	--	--
<i>Mixing by sonicated plus mixing 2800 rpm for 6 m</i>							
high Mw-PLGA 50:50 plus hTGF-β1	0.1%	w1: 250 uL PBS plus 0.06% w/w PVA plus 10 µg hTGF-β1 o: 500 mg polymer in 5 mL EA. w2: 50 mL of EA _{sat} -W plus 15% w/w glucose	4736±2368	3287±1002	93	7	70
<i>Mixing by vortex and emulsifier 2000 rpm for 5 m</i>							

Fixing the polymer concentration at 5% w/w in the oily phase, the amount of surfactant in the external phase of the emulsion was varied from 0.1% to 0.6% w/w. These tests were performed using low Mw-PLA or PLGA (75:25) and mixing the secondary emulsions at 2800 rpm for 6 min. In these conditions, PLA emulsions had an average droplet diameter of 1792±254 nm, 1613±335 nm, and 819±110 nm, respectively, and the related particles showed a mean size of 634±88 nm, 285±45 nm, and 220±41 nm, when increased surfactant concentration of 0.1%, 0.3%, and 0.6% w/w, respectively, were used. A similar trend was observed using low Mw-PLGA, obtaining droplets sizes from 5554±555nm to 1388±259 nm at increased Tween 80 concentration values; related carriers had a mean size of 342±90 nm and 205±32 nm, respectively (see carrier size and SEM images in **Figure 16 and 17**). The average diameter of droplets and carriers decreased when the concentration of surfactant increased. These data are in agreement with those previously reported ¹⁷⁰, confirming that higher surfactant concentrations lead to a higher number of micelles, which may stabilize smaller

droplets in emulsion, and produce smaller particles. Lowering the surfactant concentration beyond 0.1% w/w of Tween 80, agglomerated droplets/particles were observed especially in the case of PLA, due to emulsion instability probably induced by extremely low amount of micelles. This observation is in agreement with previous reports¹⁸³.

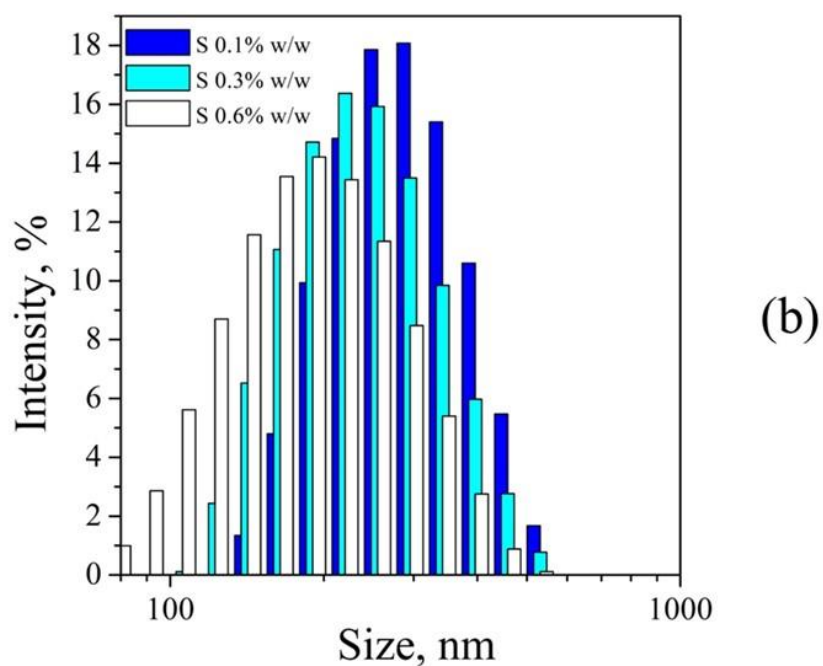
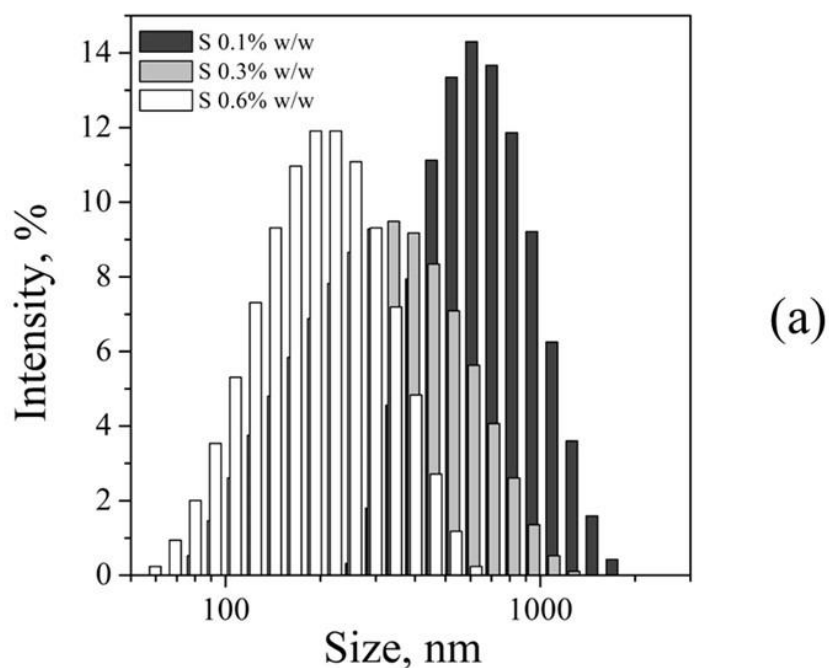


Figure 16 Influence of the surfactant content on particles size distributions.

Influence of the surfactant content in the water external phase on carrier size distribution obtained using low Mw-PLA (a) and low Mw-PLGA (75:25) (b). Fixing the polymer concentration in the oily phase and other emulsion formulation parameters, a reduction of carriers mean size was observed with the increase of surfactant concentration in the external water phase.

Shrinkage was observed between the droplets and the solid particles in all experiments performed (see also **Figure 15a** and **Table 3**). In detail, Shrinkage Factor (SF) ranges between 94-65% were measured when Tween 80 concentration varied from 0.1 to 0.6%. Generally, when fixing the polymer concentration in the oily phase (i.e., oily phase viscosity), a larger shrinkage was observed in the emulsions with a lower surfactant concentration. This observation can be explained by considering that lower amounts of surfactant used in the external water phase allows the formation of a reduced number of micelle and, therefore, larger droplets are formed. Conversely, higher surfactant concentrations stabilized smaller droplets; therefore, despite the lower droplets shrinkage, smaller carrier mean size are obtained ¹⁷⁰. The size of carriers (and the related droplet shrinkage) can be influenced by the different molecular weight of polymer used in the oily phase formulation which determines the oily phase viscosity. Therefore, the use of low Mw-PLA can be responsible for the different shrinkage values compared to that of the two PLGA used (co-polymer ratio of 75:25 and 50:50) both with higher molecular weight ¹⁶².

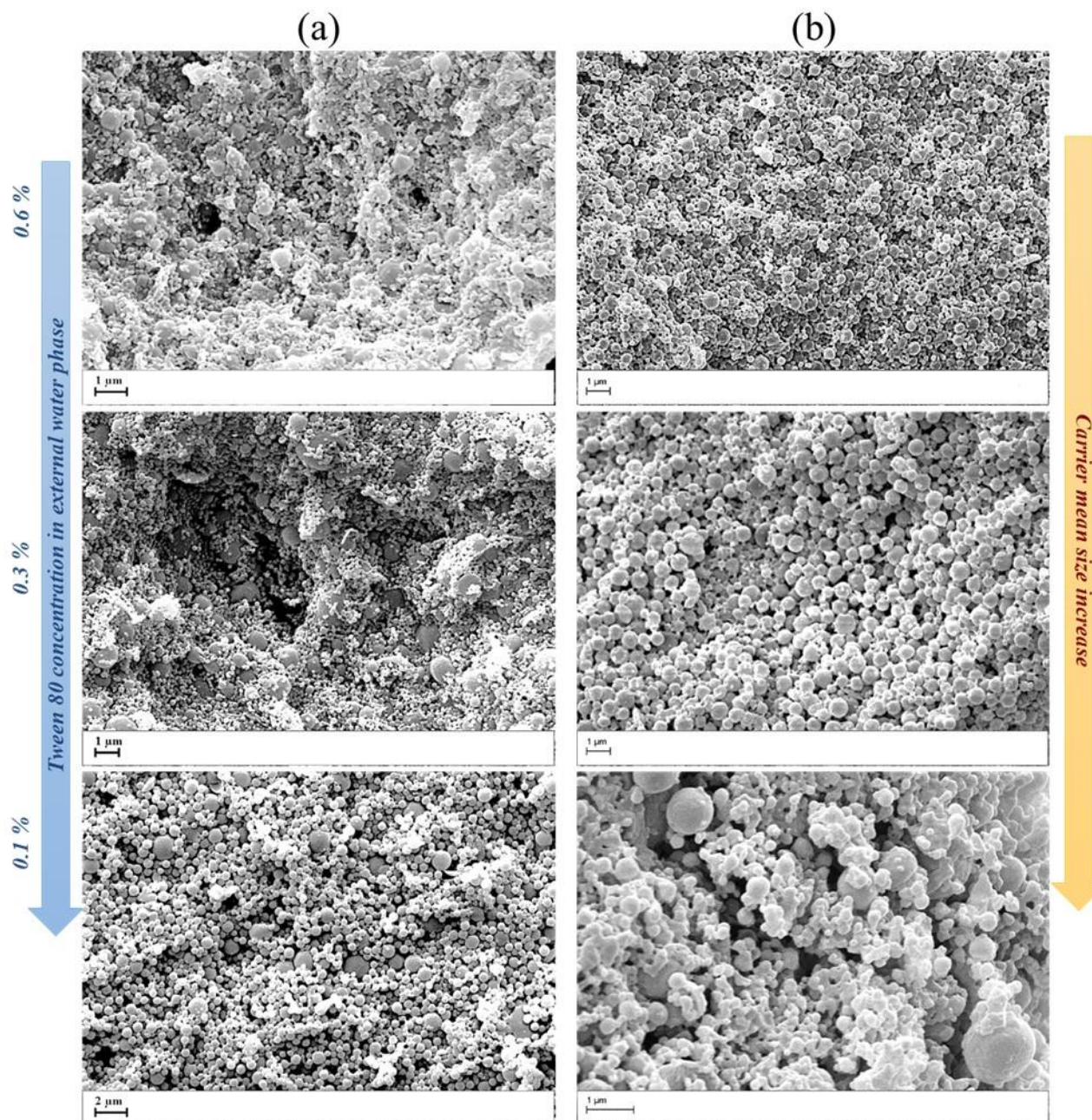


Figure 17 SEM micrographs of carriers fabricated using different emulsion formulations.

FE-SEM images of carriers fabricated using emulsion formulated at increasing surfactant concentrations in the external water phase from 0.1 to 0.6% w/w. Low Mw-PLA (a) and low Mw-PLGA (75:25) (b). Fixing all other parameters, increasing the surfactant concentration a general reduction of mean droplets size was obtained in emulsion assuring carriers mean size reduction.

Subsequently, surfactant concentration of 0.6% w/w was selected for preliminary tests of growth factor encapsulation using both low Mw-PLA and low Mw-PLGA (75:25) biopolymers. In these cases, emulsions with a droplet mean size of 858 ± 47 nm and 1653 ± 63 nm were obtained and related

carriers with a mean size of 189 ± 25 nm and 193 ± 19 nm, respectively, were fabricated. The SFs were coherent with previous data, confirming that the presence of hTGF- β 1, had no effect on emulsions stability and processing (data not shown). Preliminary tests of hTGF- β 1 encapsulation were performed using the inactive epitope of growth factor (i hTGF- β 1) given high cost of the active recombinant form. However, differences between the two epitopes were not relevant from the process point of view. The explored emulsion formulation conditions delivered anticipated carrier morphology and size control but ensured low encapsulation efficiencies.

Active peptide encapsulation requires milder conditions in terms of mixing times and speed during emulsion formulation. Moreover, the use of vortex (instead of ultrasound) was strongly recommended for emulsion phase mixing to avoid peptide denaturation. Sonication can induce changes in structural and thermal properties of proteins and increase the enthalpy of denaturation due to protein aggregation¹⁸⁴. Following these considerations, the emulsion preparation protocol was modified and the primary emulsion was obtained using vortex at maximum speed for 30 sec. In these conditions, all preliminary runs led to encapsulation efficiency lower than 20% (data not shown).

To improve encapsulation performance, fabricated larger particles and to reduce the osmotic gradient switched from external phase to internal water phase. To increase carrier size the surfactant concentration was reduced at 0.1 %w/w in the external water phase and the rotational speed of emulsifier was downgraded to 2000 rpm for 5 min. Furthermore to obtain an osmotic gradient, to improve growth factor entrapment efficiency¹⁸⁵, PBS 1X solution was adopted as inner water phase¹⁸⁶, whereas, external water phase was prepared using 0.1% w/w of Tween 80 plus glucose (15% w/w). Additionally, a PLGA co-polymer ratio of 50:50 with higher molecular weight (3,8000-54,000 Da) was selected, to increase oily phase dynamic viscosity, that may also help the encapsulation efficiency¹⁷⁰. Adopting these new conditions, larger droplet mean sizes were obtained with a consequent increase of derived carriers that showed a size of 3287 ± 1002 nm. However, the

encapsulation efficiency was increased to 70%, with carrier loading of 7 $\mu\text{g/g}$ for hTGF- β 1 (see **Table 3** and **Figure20**), respectively.

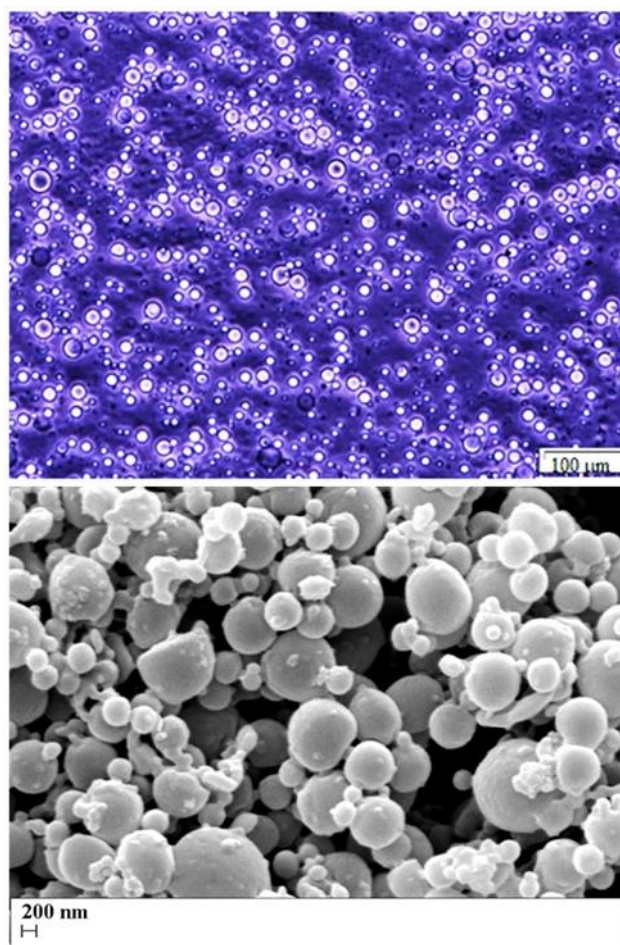


Figure 18 Optical and SEM micrographs of carriers loaded with hTGF- β 1.

Optical image of the emulsion and SEM micrograph of related carriers loaded with a growth factor; high Mw-PLGA (50:50) loaded with hTGF- β 1.

3.4.3 Comparison between SEE and SE

Using high Mw-PLGA (50:50) and fixing the emulsion composition (see **Table 4**), the same system was processed by both SEE and SE techniques. SEE technology allowed the formation of carriers with a smaller size (843 ± 226 nm) and distribution, when compared to the ones obtained by SE (1780 ± 1001 nm), as also illustrated in **Figure 19**. The results confirmed previous data^{157,158} and are explained by droplet aggregation (Ostwald maturation) during SE, which lead to a larger carrier size.

This phenomenon is prevented by the SEE process due to the fast elimination of the solvent in the oily phase and consequent rapid polymer hardening.

Table 4 Emulsion formulation processed by both SEE and Solvent Evaporation (SE) technique.

Polymer	Tween80 % w/w	Emulsion composition	DSD ($\pm$ SD) nm	PSD($\pm$ SD) nm	Shrinkage %
high Mw- PLGA 50:50	0.3%	w ₁ : 250 PBS plus 0.06% w/w PVA and human Serum Albumin (1 % w/w) o: 500 mg polymer in 5 mL EA. w ₂ : 50 mL of EAsat-W plus 15% w/w glucose	8729 $\pm$ 273	SE 1780 $\pm$ 1001	80
				SEE 843 $\pm$ 226	90

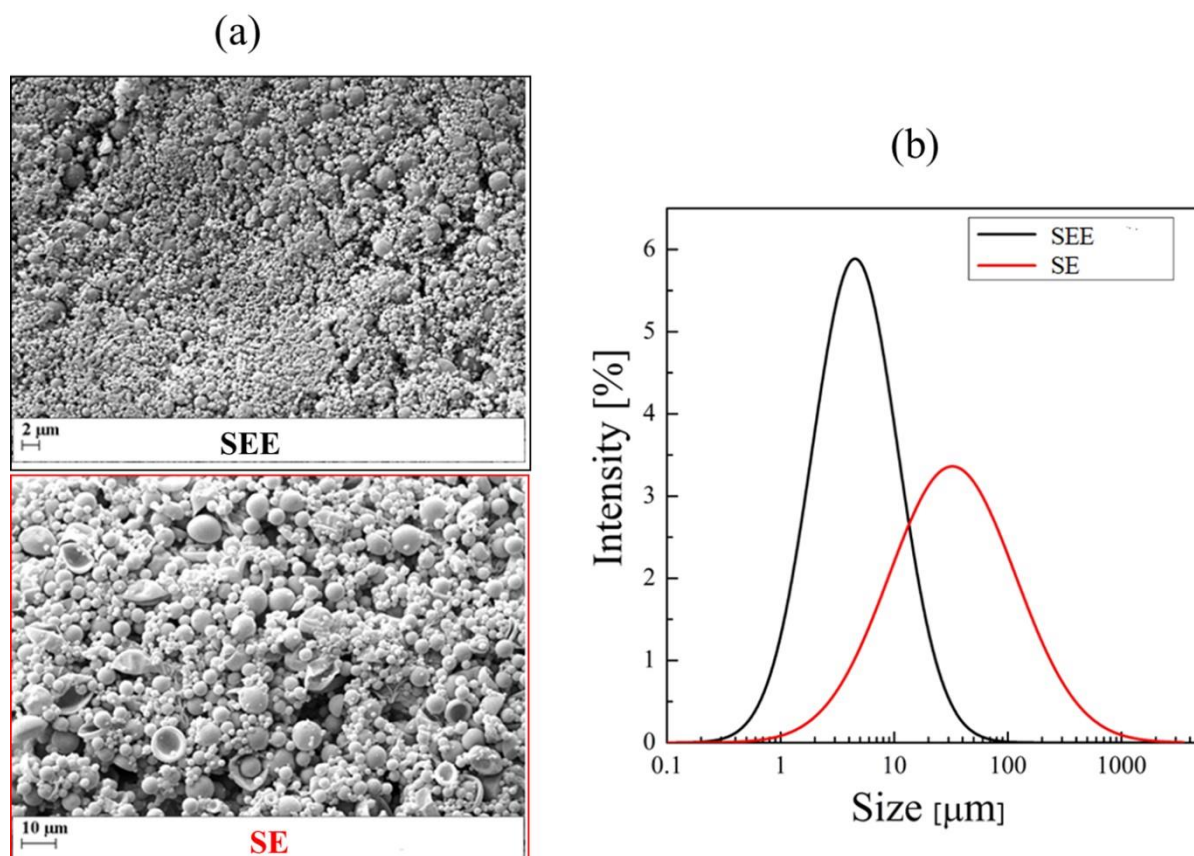


Figure 19 SEM micrographs and particles size distribution

SEM images and related Particle Size Distributions of high Mw-PLGA (50:50) empty carriers fabricated using the same emulsion processed with different technologies: Supercritical Emulsion Extraction (SEE) and traditional solvent evaporation (SE) techniques, respectively. Larger carriers size and distribution were observed in the case of particles obtained by SE.

3.4.4 Release Profile study

The release profiles are related to the amounts of peptide (ng) released from 100 mg of carriers. In the case of PLGA/hTGF- β 1 (**Figure 20**), a typical biphasic delivery profile was observed, with an initial burst release followed by a more controlled secondary release phase. The burst release of 35 ng was followed by a sustained release of about 40/50 ng/day for the following 9/10 days; the first asymptote was reached at about day 11 and 12, then, a further drug release of about 20 ng/day was observed for the following 25 days. The initial burst effect could represent the release of the growth factor entrapped on the surface of the carrier, while the drug delivery during the second phase of

release (after Day 13) likely results from polymer degradation, a typical characteristic of these PLGA systems¹⁸⁷.

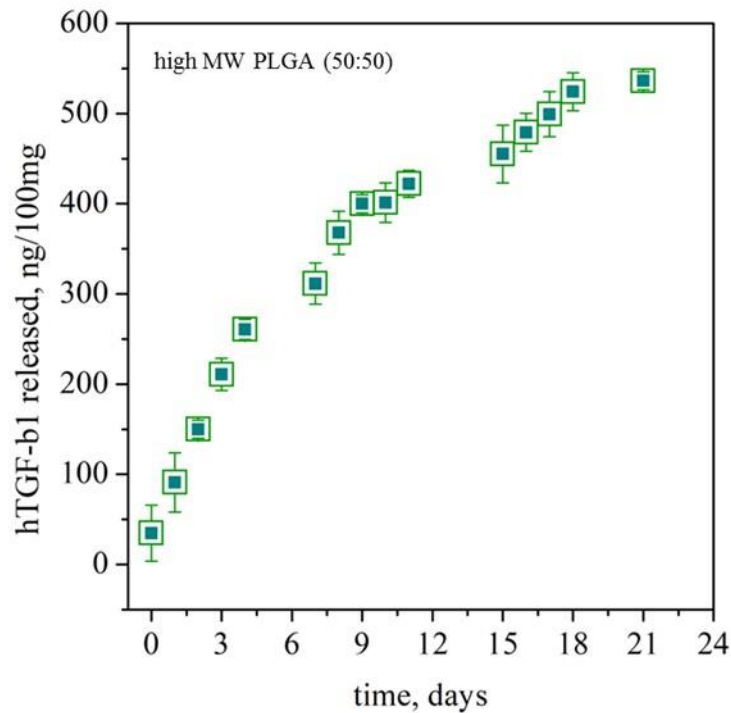


Figure 20 Kinetic characterization of hTGF-β1 loaded microcarriers

Release profile of growth factor measured in PBS at 37°C up to 25 days. The data are expressed as amounts (ng) released from 100 mg of carriers: hTGF-β1 from high-Mw PLGA (50:50) carriers;

3.4.5 Cytotoxicity studies

The cytotoxicity of carriers was determined using CHO-K1 cells. Cells were incubated for 24 and 48h using 10 µg/uL, 5 µg/uL, 2.50 µg/uL, and 1.25 µg/uL concentrations of carriers (unloaded and loaded with growth factor, **Figure 21**), and their viability was analyzed using MTT. Carriers were designed with different loadings to be enclosed within a 3D environment and commit stem cells towards a specific phenotype, the concentrations were selected according to the amounts required within the 3D environment¹³⁸.

After 24h, at the lowest concentrations, cell viability decreased up to a maximum of 50% with PLGA unloaded treatments, while PLA treatments displayed less toxicity. Furthermore, higher concentrations of both were less toxic with the highest (but not statistically significant) cell viability at 10 $\mu\text{g}/\text{uL}$, where cell viability reached about 60% at 48h. Similarly, PLGA carriers seemed to affect cell viability at lower, but not higher, concentrations (**Figure 21a**). Moreover, at all-time points explored, PLGA carriers fabricated with conventional SE seemed to exhibit more cytotoxicity than SEE PLGA carriers, originating from the same emulsion, as illustrated in **Figure 20b**. This effect may result from the reduced presence of organic solvent residue in the carriers fabricated with the SEE technique¹⁷⁶. On the other hand, treatment with pure growth factor, at the concentrations of 0.8 $\mu\text{g}/\text{uL}$; 0.6 $\mu\text{g}/\text{uL}$; 0.1 $\mu\text{g}/\text{uL}$, did not affect cell viability, stimulating cell viability and proliferation, (**Figure 21c**). At 24 and 48h, all hTGF- β 1 treatments increased cell viability compared to untreated cells. The treatment of proliferating CHO-K1 cells with hTGF- β 1/PLGA carriers exhibited a cytotoxic effect which did not exceed the effect observed for the empty carriers used at the same concentrations (**Figure 21c**). This suggests that the effect probably resulted from PLGA and not the hTGF- β 1 loaded carriers. The concentration of pure growth factors was chosen based on loading efficiency of carriers.

Higher cytotoxicity was induced by lower concentrations of carriers, likely because an interference of carrier material with specific cellular receptors and/or internal proteins involved in cell viability and proliferation together with a better carrier internalization, when diluted. Efficient internalization by cells has been already reported by Ciaglia et al. where increasing granular appearance of gated monocytes supports the uptake of carriers. We hypothesize that CHO-K1 cells might phagocyte carriers, affecting relative viability¹³³.

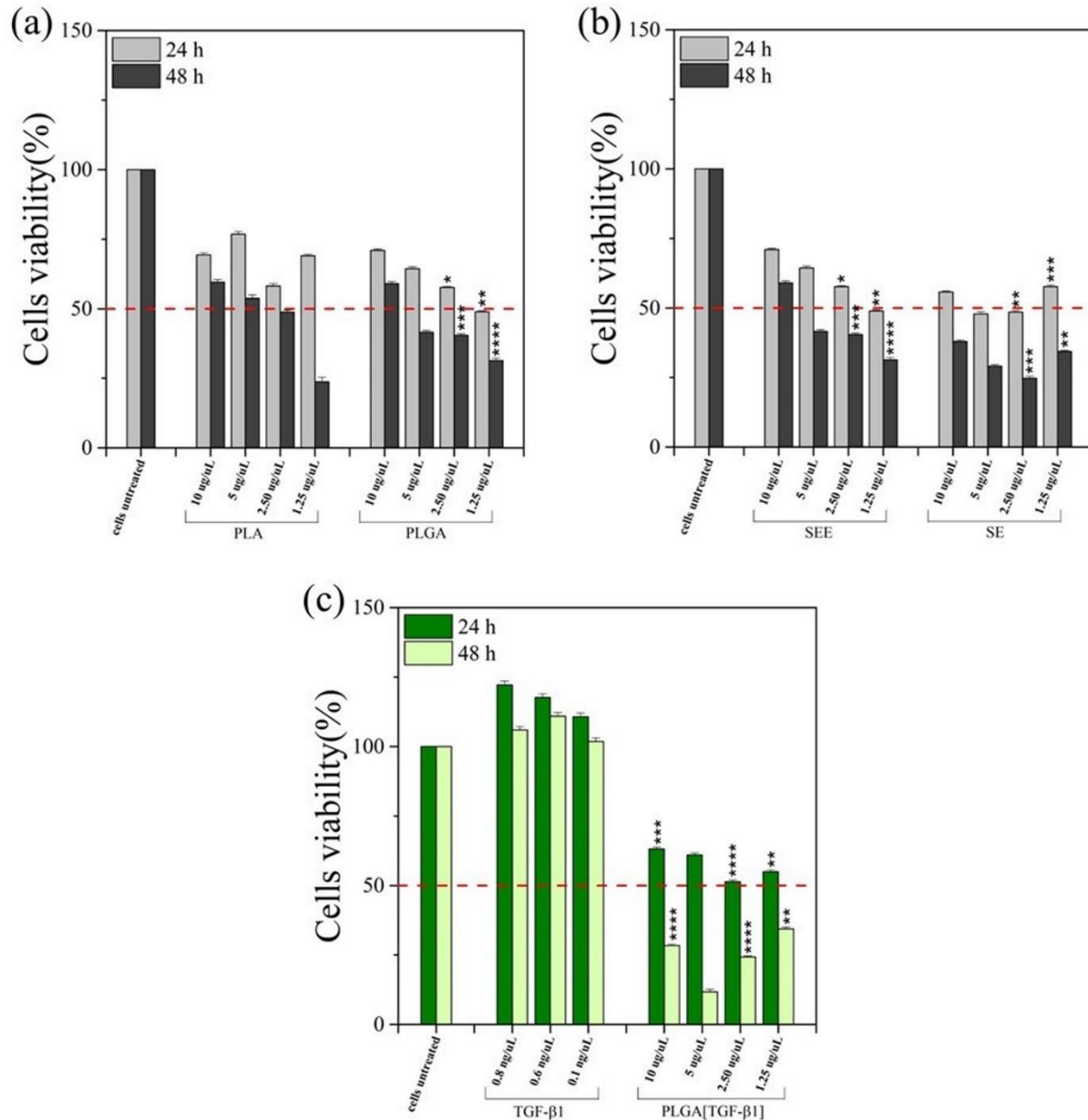


Figure 21 Carriers' cytotoxicity on Chinese hamster ovary cells.

Carriers' cytotoxicity evaluated using Chinese Hamster Ovary cell line (CHO-K1) that were treated with empty and loaded PLA and PLGA carriers for 24h and 48h: (a) empty PLA and PLGA carriers; (b) empty high-Mw PLGA carriers, fabricated with SEE and SE techniques; (c) free hTGF-β1 and hTGF-β1 loaded high-Mw PLGA carriers; The histograms report the mean percentage of viable cells compared to controls (untreated cells, 100%). The experiments were analyzed by two-tailed Student's t-test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p \leq 0.0001$; $n=3$.

Cytotoxicity of empty low-Mw-PLA and high-Mw-PLGA was also explored with hPBMCs isolated from healthy donors to provide further evidence on their toxicity directly on human primary cells. To this aim, cells were incubated for 24 and 48h with decreasing concentrations of unloaded carriers,

and viability was analyzed by MTT assay as in the CHO cell systems. No cytotoxicity was detected in hPBMCs versus controls at either 24 or 48 h of treatment with empty carriers (see **Figure 22**), though PLA carriers seemed to induce a greater increase in viability than PLGA carriers. However, for both materials, lower concentrations significantly improved cell viability suggesting a better uptake of the carriers with a significant enhancement of cell metabolism consistent with previous reports ¹⁷⁶. However, in contrast to CHO cells, neither PLGA nor PLA carriers negatively affected cell survival but instead have resulted in modest increases. This suggests that both empty and loaded carriers, especially at lower concentrations, could stimulate cell proliferation by acting as mitogens or at a minimum improving mitochondrial metabolism. Therefore, the observed effects could be explained by either a stimulating effect on mitochondrial activity of the carriers or by a more general action of these compounds on hPBMCs survival, ultimately leading to an improvement of hPBMCs metabolism. These data are in agreement with those already reported by *Da Silva et al.* who investigated PLA carriers' cytotoxicity on hPBMCs and showed an increase of cell viability of about 70% ¹⁸⁸.

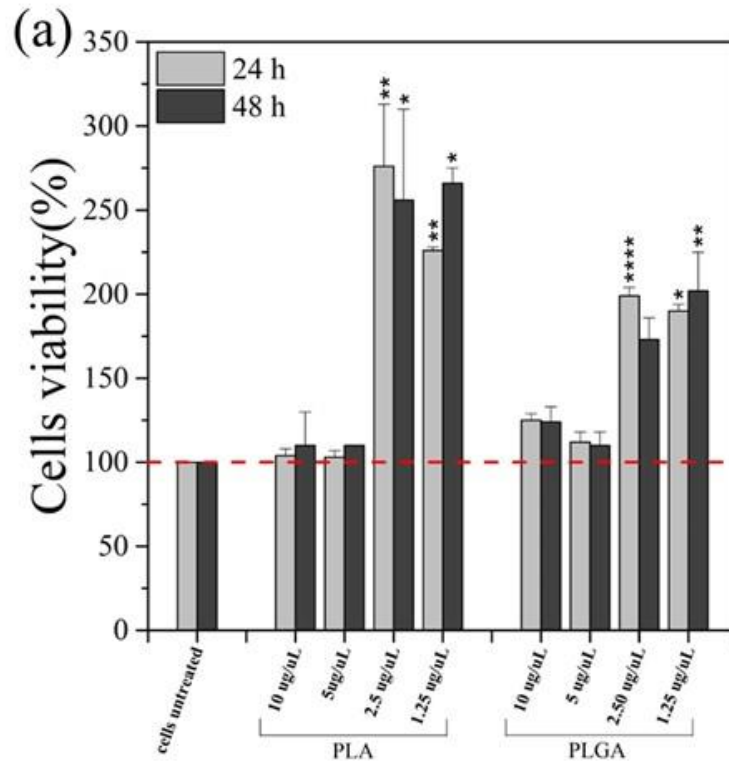


Figure 22 Carriers' cytotoxicity on human Peripheral Blood Mononuclear Cells.

Carriers' cytotoxicity was evaluated using human peripheral blood mononuclear cells (hPBMCs) that were treated with decreasing amounts of empty low-Mw PLA and high-Mw PLGA carriers for 24h and 48h: (a); The histograms report the mean percentage of viable cells compared to controls (untreated cells, 100%). The experiments were analyzed by two-tailed Student's t-test * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p \leq 0.0001$; $n=3$.

2.6 Conclusion

SEE technology can successfully encapsulate peptides (e.g. growth factor) into PLGA carriers providing advanced control of particle size and morphology, as well as ensuring sustained growth factor release over 25 days. SEE-fabricated carriers do not present intrinsic toxicity, and therefore they can be safely introduced within biomedical devices as they are unlikely to pose a risk for human health. The present investigation opens new perspectives for the use of SEE fabricated carriers in 3D bioengineered microenvironments where biological molecules can be released under controlled conditions stimulating tissue regeneration and healing.

**CHAPTER 4: Chondro-inductive injectable hydrogel:
a novel platform for cartilage healing**

4.1 Introduction

Articular cartilage injuries are a significant health problem due to their poor reparative potential. OA is caused by progressive joints wear and is often a consequence of these injuries ¹⁸⁹. The incidence of OA increases with age and is now one of the most prevalent diseases in elderly people ^{190,191}. The avascular nature of cartilage prevents spontaneous healing increasing the potential for chronic damage where conventional pharmacological therapy focuses on symptomatic treatment and pain relief, and not to regeneration of impaired cartilage ¹⁹². Patients who do not respond to pharmacological intervention ultimately require surgical procedures to promote cartilage repair, such as arthroscopic debridement, BM stimulation techniques, autologous chondrocyte implantation, and, finally, total or partial joint replacement ¹⁹³. Bone marrow stimulation requires sub-chondral level drilling to initiate bleeding, that enables mesenchymal cells to migrate from BM to the site of injury. Repaired tissue is primarily composed by fibrocartilage, with poorer biochemical and biomechanical characteristics than hyaline cartilage. Consequently, clinical outcomes are not always adequate ¹⁹⁴.

TE is alternative strategy to traditional therapeutic approaches and offers potential to improve joint cartilage therapy ¹⁹⁵. TE describes the application of biocompatible and biodegradable polymers as 3D scaffolds to support cell growth and differentiation. The scaffold has become a focus of interest where tailoring mechanical or biochemical properties to replicate native natural ECM is becoming a key goal. The ECM is itself a dynamic network providing mechanical force to support tissue function and the reproduction of its physical and biochemical properties represents a major TE challenge ¹⁹⁶. The articular cartilage ECM is composed mainly of hydrated collagen (e.g., type II) and proteoglycans (e.g., aggrecan), with high water content to assure and maintain its specific mechanical properties ¹⁹⁷.

TE scaffolds are generally composed by biodegradable and biocompatible materials, natural or synthetic, and the correct choice of scaffold is crucial to the success of therapeutic strategy ¹⁹⁸. Collagen-based scaffolds are appropriate to host chondrocytes due to their similarity to native ECM.

However, hydrogels exhibit low mechanical resistance and consequently are often cross-linked to create adequate stiffness^{142,199}. Particularly, type I collagen hydrogels are frequently investigated as being suitable for implantation given their *in vivo* biocompatibility²⁰⁰. Furthermore, cells into a 3D culture environment are subjected to lower oxygen content, indeed they receive fewer exchanges from the external medium and tend to be in a hypoxic state²⁰¹. Hypoxia has been also reported to be favorable for the hMSCs induction towards both chondrogenic and tenogenic phenotype^{202,203}.

On the other hand, induction of chondrogenic differentiation frequently depends on the supplementation of growth factors, such as TGF- β 1 and TGF- β 3. Of these, TGF- β 1 is the most widely investigated for the promotion of chondrogenesis in hMSCs and hASCs, however, its dose-response effect remains still unclear. In relation to this, hBM-MSCs cultured with a TGF- β 1 concentration ranging from 0.01 to 0.1 ng/mL revealed no evidence of chondrogenic differentiation^{121,204}. In contrast, hASCs were induced by 10 ng/mL TGF- β 1 supplementation into aggregates with the expression of sulfated proteoglycans and aggrecan, while 100 ng/mL reduced this chondrogenic phenotype²⁰⁵. Growth factors are routinely administered via a supplemented medium which has to be replaced frequently, with a high economic impact due to the relevant cost of recombinant peptides.

Recent attention has focused on the encapsulation of growth factors into biopolymeric micro/nano carriers to promote their controlled local release within 3D microenvironments for tissue engineering application^{206–208}. Micro/nano carriers fabrication can be based on emulsion processing by evaporation or extraction of the oily phase and subsequent collection of the resulting biopolymer beads. Dense gas technology has also been described for oily phase extraction, allowing accurate carrier size control and loading of several active substances^{209–211}. Carriers can be immobilized with ease within a hydrogel matrix, and the controlled delivery of their cargo within the 3D scaffold accomplished. This creates the possibility of optimized control of constant growth factor delivery within a 3D system²¹².

Dynamic conditions are an essential component of 3D culture systems^{206,213,214}. Perfusion upregulated chondrogenic markers^{63,215}, preserved the cartilage phenotype, and prevented hypertrophy of MSC-derived chondrocytes, which remains a fundamental problem in cell-based strategies⁶⁴. Furthermore, when micro and nano carriers are used for controlled delivery of growth factors, an adequate release is promoted by dynamic environment, whereas in static conditions, reduced mass transfer can decrease the extent of drug release, impacting the scaffold efficacy in driving cell commitment⁵⁴.

4.2 Aims

The present study aimed to evaluate the potential of 3D collagen microcarrier functionalized scaffolds, as a bioengineering tool for induction and maintenance of chondrogenic commitment of human stem cells. The attention will focus on fabrication of PLGA carriers for hTGF- β 1 controlled release, the assembly of 3D scaffold systems with hBM-MSC and their 3D culture along 16 days. Gene expression profiling by qRT-PCR and histological and qIF analysis will provide indications on cells behavior at given time points, and cytokine gene expression can suggest the cell reaction against 3D environment in both static and dynamic conditions.

4.3 Materials and Methods

4.3.1 hBM-MSC isolation and harvesting

hBM-MSCs were obtained from bone marrow of independent healthy donors (male, age 40 and 38), after written informed consent obtained in accordance with the Declaration of Helsinki to the use of their residual bone marrow aspirate for research purposes, with approval from the University Hospital of San Giovanni di Dio e Ruggi d'Aragona (Salerno, IT). Review Board (prot./SCCE n. 24988 achieved on April 09th, 2015).

Briefly, total BM aspirate was directly seeded at a concentration of 50,000 total nucleated cells/cm² in T75 plastic flask in α -MEM supplemented with 1% GlutagroTM, 10% FBS and 1% Penicillin/Streptomycin (Pen/Strep) and incubated at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity ²¹⁶. After 72h, non-adherent cells were removed by medium change and the remaining adherent cells were then fed twice weekly thereafter with new medium. On day 14, colonies of adherent hBM-MSCs were detached and re-seeded at 4000 cells/cm² in the same culture conditions. Once the cell cultures reached 70–80% confluence (7-8 days from the previous passage), cells were detached using 0.05% trypsin-0.53mM EDTA and washed with 1X of Phosphate buffered saline (PBS) (Corning Cellgro, Manassas, VA, USA), counted using Trypan Blue (Sigma-Aldrich, Milan, IT) and subcultured at a concentration of 4×10^3 cells/cm². At Passage 2, cells were used experimentally. Flow cytometry analysis was performed on hBM-MSCs obtained at Passage 2; cells were positive for CD90, CD105, CD73 and negative for CD14, CD34, CD45, HLA-DR expression (Miltenyi Biotec, DE) (data not shown) ²¹⁷.

4.3.2 PLGA-MC fabrication by SEE

PLGA-MCs were obtained using Supercritical Emulsion Extraction (SEE) technology via dense gas extraction of emulsion oily phase. The process layout is described by a countercurrent packed tower operating in continuous mode ²¹⁸. PLGA-MCs were fabricated using a *water-oil-water* emulsion (ratio 0.25:5:50). In detail, recombinant hTGF- β 1 (PeproTech; UK) was dissolved into 0.1% w/v human serum albumin (hSA; Sigma-Aldrich, Milan, IT) containing 0.06% w/w of polyvinyl alcohol (PVA; Sigma-Aldrich, Milan, IT) as surfactant, hSA was included as growth factor stabilizer in the primary emulsion ²¹⁹. The water phase was added to the oily phase formed in ethyl acetate (EA, purity 99.9%) and PLGA (50:50; RESOMER[®] RG 504H, 0.45-0.60 [dL/g] from EVONIK) at 5% w/w. The water/oil (w/o) emulsification was produced by vortex mixer for 30 s at maximum speed with the

resulting primary emulsion immediately poured into an EA-saturated aqueous solution containing 0.1% w/w Tween 80 and 15% w/w glucose. The secondary emulsion was then formed via a high-speed homogenizer (mod. L4RT; Silverson Machines Ltd., Waterside, Chesham Bucks, UK) at 2000 rpm for 5 min and at 10°C. All emulsions were processed by SEE immediately after their preparation. Operative pressure and temperature conditions in the high-pressure column were set at 8 MPa and 38°C, respectively, with a dense gas flow of Carbon Dioxide (CO₂) set at 1.4 kg/h with Liquid/Gas ratio of 0.1 w/w²²⁰. PLGA-MC suspensions were collected at the bottom of the extraction column, washed to eliminate surfactant, and lyophilized. All washing steps were performed in sterile conditions supplemented with a pen/strep and amphotericin B (1% w/v) solution.

4.3.3 Carrier size distribution and morphological analyses

Particle size distributions (PSDs) of PLGA-MC suspensions were measured using laser granulometer (mod. Mastersizer S; Malvern Instruments Ltd., Worcestershire, UK), based on dynamic light scattering (DLS). Sizes are expressed as volume mean (MS) with standard deviation (SD) in nanometers (nm). The shape and morphology of the PLGA-MCs were investigated by field emission-scanning electron microscopy (FE-SEM, mod. LEO 1525; Carl Zeiss, Oberkochen, D). PLGA-MC samples were placed on double-sided adhesive carbon tape previously glued to an aluminum stub and coated with a gold film (250 Å thickness) using a sputter coater (mod.108 Å; Agar Scientific, Stansted, UK).

4.3.4 hTGF-β1 release study

The release profile of hTGF-β1 from PLGA-MCs was monitored in vitro for 21 days from 5 ± 0.3 mg of PLGA-MCs suspended in 500 µL of PBS 1X containing 0.1% Tween 20 and 0.1% hSA, placed in an incubator at 37°C, and stirred continuously at 100 rpm. Every 24 hours, the samples were

centrifuged at 14000 rpm for 30 min, and the supernatant completely removed and replaced with fresh PBS to maintain sink conditions. Released hTGF- β 1 concentrations from collected samples were then measured by ELISA, (Cloud-Clone Corp., USA). Experiments were performed in triplicate (N=3), and curve describing the mean profile were calculated as ng/g (protein released/PLGA-MCs) versus time or as a percentage amount calculated with respect to the maximum growth factor load.

4.3.5 3D collagen scaffold preparation and characterization

For each scaffold, a mixture of type I collagen derived from rat tail (Advanced BioMatrix; US), neutralization solution (Advanced BioMatrix; US), and α -MEM (Corning, NY, USA) was added at a 9:1:2 ratio. This was enriched via addition of 22 mg/mL of PLGA-MCs (7 ug/g hTGF- β 1/PLGA) and 1×10^6 cells/mL creating a scaffold mastermix. A 300 μ L volume of mastermix was pipetted drop by drop into cylindrical molds (5x5 mm diameter vs. height) then placed into an incubator at 37°C for 60 min to allow collagen thermal cross-linking. The obtained 3D bioengineered scaffolds (0.3% w/w collagen) were then transferred into culture plates.

Cell viability in scaffolds was detected by fluorescence live/dead assay (Calcein AM solution (Cat. no C1359) and Ethidium homodimer I solution (Cat. no E1903), Sigma-Aldrich, Milan, IT), immediately after making scaffold. Cells were stained for 1 hour at 37°C, washed in PBS 1X and imaged in a fluorescence microscope (mod. Eclipse, Nikon, DE).

Internal morphology of the scaffold was observed by FE-SEM (mod. LEO 1525; Carl Zeiss, Oberkochen, DE). Samples were first fixed in 4% (w/v) paraformaldehyde (PFA) (4°C, overnight), and then dehydrated by multiple passages across ethanol: water solutions (10 minutes each) with increasing percentages of ethanol (10%, 20%, 30%, 50%, 70%, 90%), ending in a 100% dehydrating liquid (3 changes, 10 minutes each), and lyophilized using a critical point dryer (mod. K850 Emitech, Assing, Rome IT). Samples were first immersed in liquid nitrogen until freezing, and then fractured

with a needle in order to expose their internal surface, placed on a double-sided adhesive carbon tape previously glued to an aluminum stub, and coated with a gold film (250 Å thickness) using a sputter coater (mod.108 Å; Agar Scientific, Stansted, UK), before observation.

4.3.6 Static and dynamic culture

For static condition, 3D scaffolds were placed into a multiwell plate, while for the dynamic stimulation, a custom perfusion bioreactor was used. The bioreactor was formed by a multiwell plate milled in poly(methyl methacrylate) (PMMA, Altuglas® CN 100 10000, Altuglas International, La Garenne-Colombes Cedex, FR), a biocompatible material for biomedical applications²²¹. The plate has two holes allowing insertion of silicon tubes (Tygon®, FR) for flow provision via peristaltic pumps at a constant flow rate of 1.0 mL/min²²². This continuous flow was maintained during dynamic experiment. The chondrogenic media was recycled from the peristaltic pump and changed twice a week with fresh. The bioreactor system operated within a standard cell culture incubator.

4.3.7 Hematoxylin and eosin and Sirius red staining

Scaffold sections (15 µm thickness) were hydrated using a decreasing ethanol gradient to 75% and then washed for 5 min in water. Hematoxylin and eosin staining and Picrosirius Red Stain Kit (Polysciences, Inc.) were used for 60 min. Samples were dehydrated using an increasing ethanol gradient and cleared in xylene for 5 min. Finally, sections were mounted using Eukitt (Sigma-Aldrich, Milan, IT) mounting medium. Picrosirius red brightfield and polarized light images were acquired with a Brunel polarization microscope equipped with a Nikon D500 camera.

4.3.8 Immunofluorescence assay

Collagen scaffold was fixed in 4% paraformaldehyde (PFA) for 2h at room temperature, cryo-protected in 30% sucrose (4°C, overnight), included in optimal cutting temperature (OCT) compound,

and cut in slices of 15 μm of thickness using a cryostat (mod. CM1950, Leica, Wetzlar, Germany). Slices were permeabilized with 0.1% Triton X-100 for 10 min and blocked with Bovine Serum albumin (BSA) solution (1% w/v) for 1h. For type II and type III collagen staining, slices were incubated overnight at 4°C with a rabbit polyclonal anti-collagen type II antibody (1:100, Abcam, UK) and mouse polyclonal anti-collagen type III antibody (1:100; Santa Cruz Biotech., USA). Following incubation with the primary antibody, slices were incubated for 1h at RT with the Alexa FluorTM 488 goat anti-rabbit IgG (1:400, Thermo Fisher Sci., USA) and the DyLight 649 anti-mouse IgG (1:500, BioLegend, CA, USA) antibodies. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). For type I collagen staining, slices were incubated overnight at 4°C with a rabbit polyclonal anti-collagen type I antibody (1:100, Abcam, UK); then, Alexa FluorTM 488 goat anti-rabbit IgG (1:400, Thermo Fisher Sci., USA) antibody was used.

Single images were acquired with identical light intensity, exposure time and gain settings, using an inverted Leica laser-scanning confocal microscope (mod. TCS SP5; Leica Microsystems, Wetzlar DE) equipped with a plan Apo 63X/1.4 NA oil immersion objective. Signals intensity was quantified using the software ImageJ (rel.1.52p National Institutes of Health, USA). Original images in RGB format were converted into a 16-bit (gray scale) format. Thereafter, the tagged areas were expressed as an average value of pixel intensity within a range from 0 (dark) to 255 (white). Data were normalized to the number of cells present in the whole field⁹⁴. Ten images of several fields were used for the analysis at each time point. All data were reported as fold change relative to untreated cells.

4.3.9 RNA isolation and gene expression profile

Total RNA was extracted from *hBM*-MSCs seeded into both 12 well-plates and collagen scaffolds using QIAzol® Lysis Reagent (Qiagen, DE), chloroform (Sigma-Aldrich, Milan, IT) and the RNeasy Mini Kit (Qiagen, DE). For each sample, 1 μg of total RNA was reverse transcribed using the

iScript™ cDNA synthesis kit (Bio-Rad, Milan, IT). Relative gene expression analysis was performed in a LightCycler® 480 Instrument (Roche, IT), using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) with the validated primers for COL1A1, COL2A1, COL3A1, SOX9, ACAN (Bio-Rad, USA) and for pro-inflammatory cytokines IL-6, TNF, IL-12A, IL-1 β and anti-inflammatory ones IL-10, TGF- β 1 (Bio-Rad, USA) following MIQE guidelines⁹². Amplification was performed in a 10 μ L final volume, including 2 ng of complementary DNA (cDNA) as template. The specificity of the formed products was addressed via melting curve analysis. Triplicate experiments were performed for each condition explored, and data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (reference gene), applying the geNorm method⁹³ to calculate reference gene stability between the different conditions (calculated with CFX Manager software; $M < 0.5$). Fold changes in gene expression were determined by the $2^{-\Delta\Delta C_P}$ method and are presented as relative levels versus untreated cells at each time-point explored.

4.3.10 FEM modeling

Finite Element Modeling (FEM) was implemented using COMSOL Multiphysics Software to assess nutrient consumption and waste production during the cell culture. Mass transport based on the second Fick law was modeled for both static and dynamic regimes, where all components were obtained using primitive geometries and Boolean operations that considered both well and scaffold to have a cylindrical geometry (well = 22 mm in diameter and 7.4 mm in height; scaffold 5 mm in diameter and 5 mm in height). Assumed boundary conditions were laminar flow and mass transport with a permeability coefficient of hydrogel assumed as $2 \cdot 10^{-9} \text{ cm}^2$ ²²³. Other mass transport parameters are reported in **Table 5**. At steady-state conditions, the concentrations of nutrient (consumed) and waste (produced) variation was simulated taking into account a flow rate of 1 mL/min and considering the different diffusion parameters across the medium or across the hydrogel. A

sensitivity study of the mesh addressed the most computationally efficient solution. The boundary conditions, constants and main parameters used in the model were derived from previous FEM simulation data ²²².

Table 5 Mass transport parameters

FEM modeling parameters	Values	Units
Glucose at time zero	0	mol/m ³
Waste at time zero	0	mol/m ³
Nutrient consumption	$4.74 \cdot 10^{-12}$	mol/s
Waste production	$3.4 \cdot 10^{-12}$	mol/s
D coeff nutrients	$3.9 \cdot 10^{-9}$	m ² /s
D coeff waste	$1.6 \cdot 10^{-9}$	m ² /s
K collagen hydrogel	$2 \cdot 10^{-9}$	m ²

4.3.11 Statistical analysis

Results, obtained from multiple experiments (N=3 biological replicates), are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using the two-tailed independent Student's T test for comparisons of two independent groups. To compare the influences of the chemical and dynamic stimulation within the same time point, two-way ANOVA was used followed by Tukey's multiple comparison test. P values less than 0.05 were accepted as significant ²²⁴. All statistical analysis was conducted using GraphPad Prism software (6.0 for Windows).

4.5 Results and discussion

4.5.1 PLGA carrier characterization and hTGF- β 1 release profile

PLGA-MCs were fabricated by proprietary technology utilizing dense gas (supercritical carbon dioxide) to provide a micrometric sized system for the local sustained release of hTGF- β 1 ⁵⁴. PLGA-MCs displayed a spherical shape with a mean size of $3 \pm 0.9 \mu\text{m}$ (**Figure 23a,b**), but they were not suitable for conventional sterilization methods because could cause biopolymer oxidation or

degradation of peptide payload. To avoid these issues, a specific SEE operational protocol for sterile materials production was applied, which allowed recovering carriers in a sterile suspension ²⁰⁸. Carriers were fabricated with an hTGF- β 1 loading of 7 μ g/g that provided a sustained release profile over 21 days into PBS at 37°C (**Figure 23c**). A total load of 6.6 mg within each 3D collagen scaffold provided an actual hTGF- β 1 release as illustrated in **Figure 23d**. The release at day 1 was 4.6 ng/mL followed by a mean concentration of about 7 ng/mL per day for the next 6 days. A concentration of 3.3 ng/mL/day across the following days was maintained, except at day 15, when a spike of 4.5 ng/mL resulted from PLGA initial depolymerization activity ¹⁴⁸. The release data profile was only reflective of the 3D scaffold when maintained in a sink condition provided *in vitro* by continuous stirring or perfusion. When the same carriers were maintained in a static environment without medium replacement, the hTGF- β 1 release was not observed or completely underestimated (data not shown). As a consequence, we supposed that the growth factor was not properly released within the 3D scaffold maintained in static conditions along the culture period; conversely, the dynamic conditions by perfusion would better mimic the sink conditions required for proper drug release from PLGA carriers.

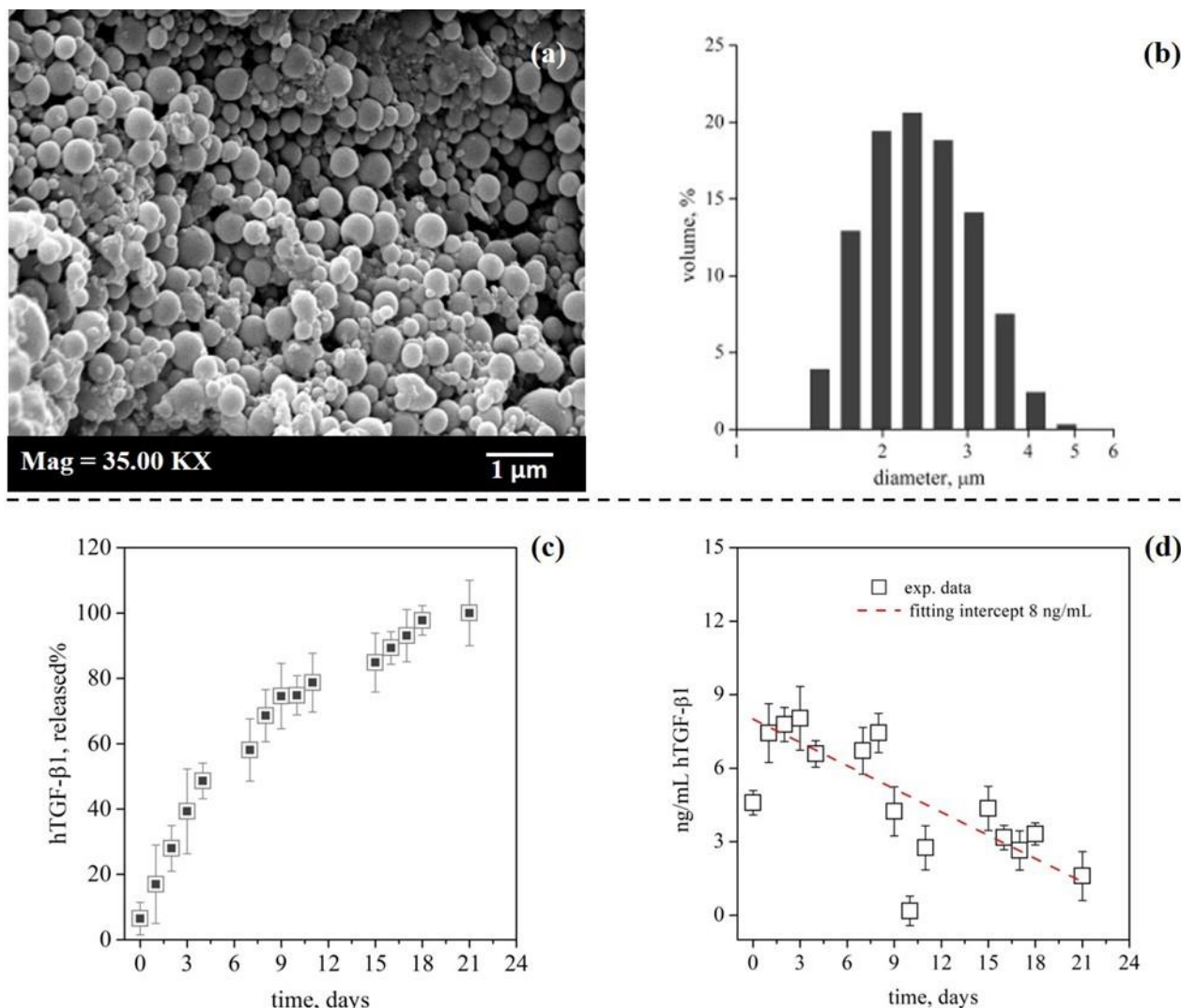


Figure 23 Characterization of carriers: morphology, particle size distribution and hTGF- β 1 release profiles for PLGA-MCs

SEM image (a) and particle size distribution (b) of PLGA carriers obtained after SEE process; size distribution data are expressed as volume percentage. hTGF- β 1 release profiles expressed as percentage of total load and monitored at 37°C and 100 rpm by ELISA-based assay; $n = 3$ (c); hTGF- β 1 amount (ng/mL/day) released within each 3D-scaffold loaded with 6.6 mg of carriers in dynamic condition (d).

4.5.2 3D scaffold environment assembly and characterization

A schematic representation of the 3D scaffold assembly protocol and resulting composite hydrogel is provided (Figure 24a, b). To better understand the impact on scaffold architecture of co-seeding with carriers and cells we performed additional characterization. PLGA-MCs loaded with rhodamine B (Rod-B, loading about 8 mg/g) and hBM-MSCs were co-seeded in a scaffold assembly to observe

carriers and cells dispersion. Dispersed elements, tracked by fluorescence where DAPI was used to highlight cell nuclei, demonstrated a homogeneous distribution within the scaffold (**Figure 24c**). A potential issue was that during mastermix extrusion, the distribution of velocity and shear stress across the cross-section of the pipette nozzle would stress the cells loaded within the gel system, particularly at the wall areas ²²⁵. We confirmed hBM-MSCs viability, following on from collagen cross-linkage, to be 97% (**Figure 24d**) which was reduced slightly to 90% following on from dynamic culture and further reduced to 80% in the static environment.

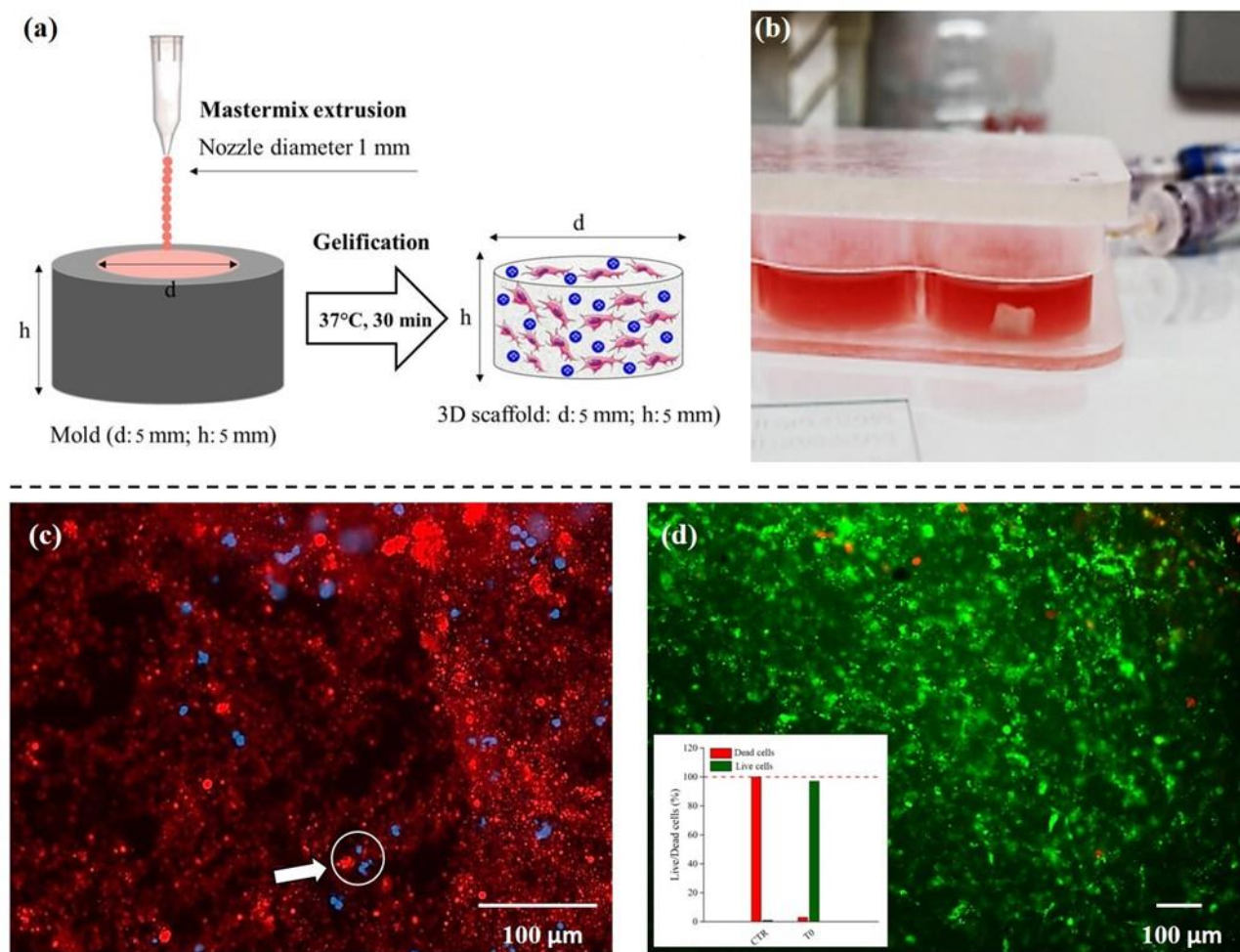


Figure 24 Production and characterization of the functionalized 3D scaffold.

Schematic illustration of extrusion and gelification process of the collagen scaffold **(a)**; Image of the 3D system within the bioreactor after 16 days of culture **(b)**. Confocal image of the scaffold integrated with hBM-MSCs (DAPI nuclei highlighted in blue) and PLGA-MCs loaded with Rhodamine B (highlighted in red) **(c)**; Live/dead signal evaluation of hBM-MSCs viability right after collagen extrusion and cross-linking **(d)**.

Then, collagen 3D scaffold was fixed, dehydrated, and freeze-fractured before FE-SEM images acquisition at different enlargements, where a collagen fiber network was observed **(Figure 25a)** with cells and PLGA carriers embedded within **(Figure 25b-d)**.

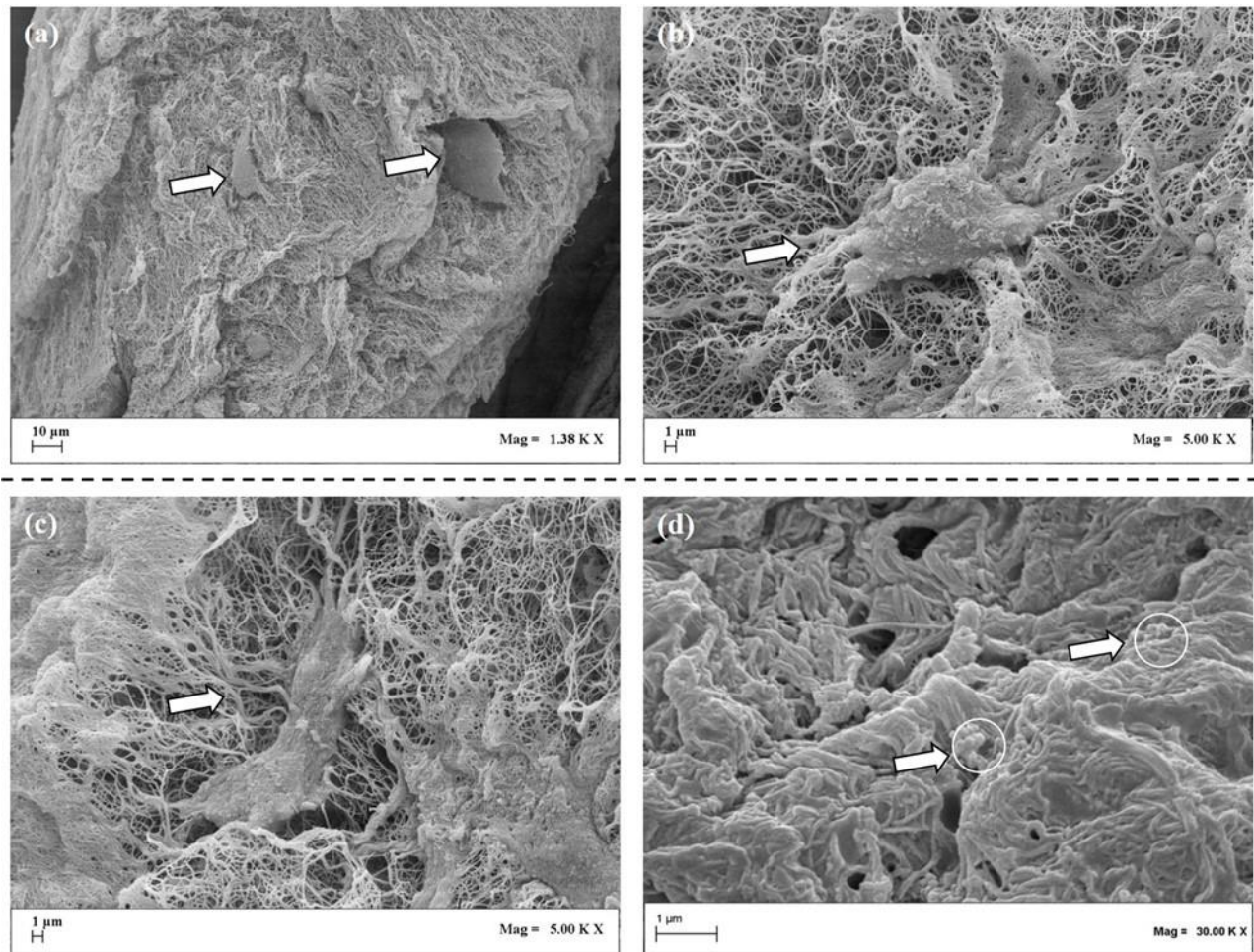


Figure 25 Freeze-dried scaffold observed by a Scanning Electron Microscope at different enlargements.

The scaffold structure is formed by a network of fibers with inside cells distributed (a); collagen fibers are organized in randomly assembled web in where cells were clearly observed (arrowheads) (b, c); PLGA carriers are embedded within the collagen fibers (arrowheads with white circle) (d).

4.5.3 Dynamic culture by perfusion bioreactor

FEM analysis of the nutrient concentration within a single culture well, in static vs. dynamic conditions, was performed (Figures 26a,b). 3D constructs were maintained in dynamic culture conditions with a 1 mL/min continuous flow rate through each well (Figure 26b,c); in this condition, a laminar flow (grey lines) and a uniform velocity distribution (horizontal cross-section) were maintained within culture wells (Figure 26d). In static conditions, the *in silico*-model demonstrated

an uneven nutrient distribution inside wells, with the lowest observed concentration in the 3D construct (**Figure 26a**). Conversely, a more uniform nutrient distribution was maintained in the whole scaffold volume in dynamic conditions, as estimated by the order of magnitude of nutrient concentration, 10^{-8} mol/m³ in static vs. 10^{-7} in the perfused environment (**Figure 26b**). The converse relationship was observed when performing waste product consideration (**Figure 26a,b**). These data reflect previous reports, where cells immobilized within a 3D system are at risk of reduced nutrient and oxygen exchange linked to impaired metabolic activity in static culture environments ^{207,226}. Moreover, overall mass exchange being favored by perfusion suggests an adequate environment to ensure appropriate sink conditions which enforce sustained release of growth factor from PLGA carriers dispersed within the 3D system ¹⁴⁸. The absence of proper mass exchange could negatively affect not only nutrient and waste exchange but also affected system behavior, preventing the achievement of an adequate release. The poor outcome of static culture, where chondrogenic markers and cytokines were down regulated, might be linked to suboptimal mass exchange that also prevented hTGF- β 1 release within the 3D system and, consequently, proper cells stimulation.

Collagen scaffold alone did not undergo any rearrangements or degradation along 16 days without cells cultured within, even if it is maintained in culture medium; furthermore, no changes in scaffold porosity, volume or color were observed in absence of cell culture (data not shown). On the contrary, the same scaffold loaded with hBM-MSC underwent a change in its volume (reduced of 1/3) and color (whiter) after 16 days.

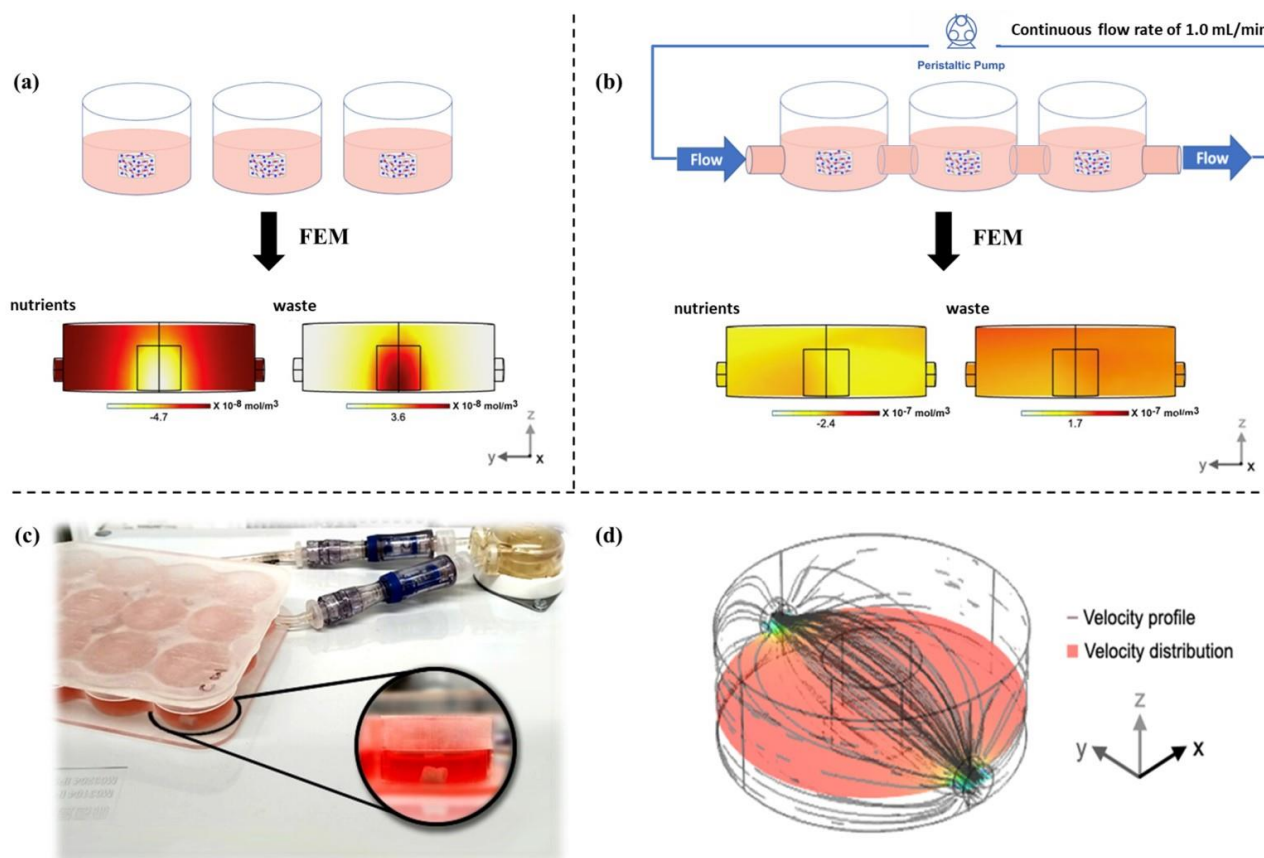


Figure 26 Perfusion culture bioreactor and FEM analysis of nutrients and waste mass exchange in static and dynamic conditions.

Schematic illustration of the static (a) and dynamic (b) environment for the 3D scaffolds culture and related FEM analysis of nutrient consumption and waste production in both static vs. dynamic condition; molecule concentrations are represented as mean value and are expressed as mol/m^3 . Perfusion bioreactor images and 3D scaffold details (c) and FEM analysis of medium velocity profile (grey lines) and distribution (horizontal cross-section) within a single culture well (the perfusion flow is oriented along the y-direction) (d).

Histological evaluation by H&E staining was performed to determine changes to scaffold composition and structure during extended culture. At day zero, cells were homogeneously distributed within the hydrogel matrix and which itself appeared randomly assembled (**Figure 27**). 3D collagen matrix maintained its integrity during the static culture and even at day 16 only small hollow areas were observed; conversely, the matrix surrounding cells underwent a greater extent of remodeling in the dynamic regime and distinct and novel regions deposition were also noted for cell-rich regions (see **Figure 27**, day 8 and 16 in dynamic condition).

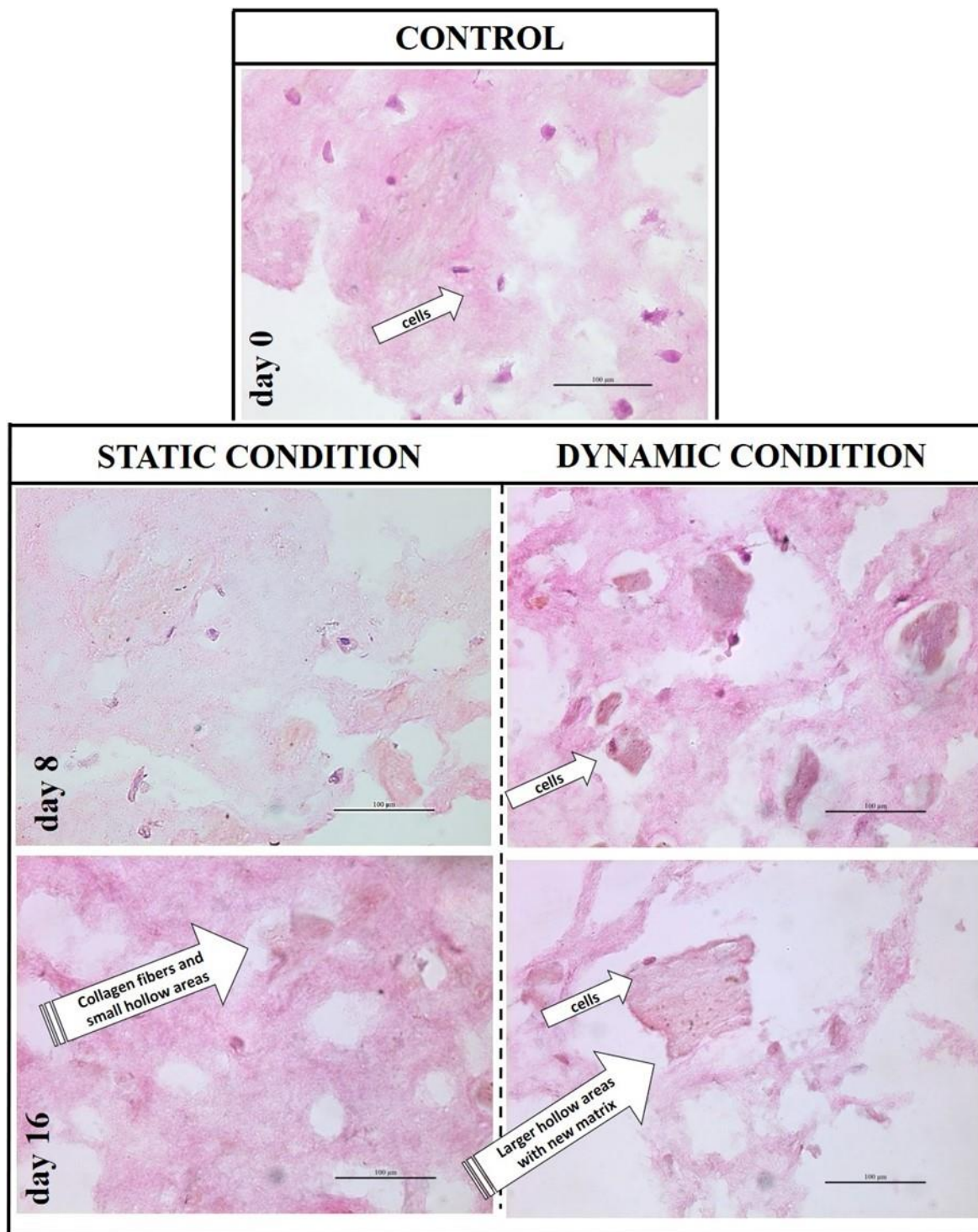


Figure 27 Hematoxylin & Eosin staining of 3D bioengineered system at different time-points in static and dynamic culture.

An homogenous network of synthetic collagen matrix at Day 0 was observed with cells immobilized within. The 3D collagen matrix maintained its integrity during the static culture; at day 16 small hollow areas were observed. On the contrary, same scaffolds cultured in dynamic condition, appeared with an increased number of hollow areas, also larger. These areas appeared filled with a darker new matrix with cells clearly observed within (arrowheads).

This behavior was also confirmed by polarized microscope observation of Sirius red stained samples illustrated in **Figure 28**. Synthetic fibers (in red) are evident along culture evolution; however, they were progressively reshaped by cells and several hollow areas appeared filled with darker red areas where cells were clearly evident. These new matrix areas were more abundant in dynamic culture and supposed to be new collagen, even if fibers appeared not yet organized.

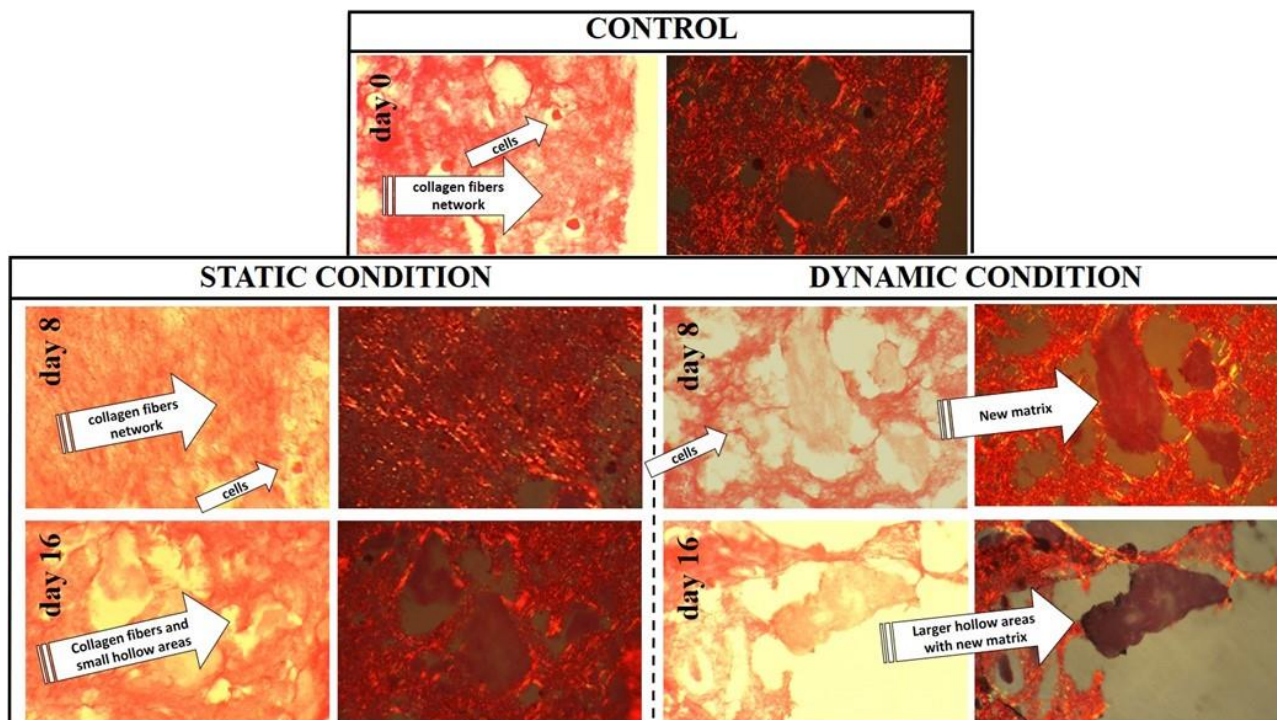


Figure 28 Polarized microscope images of 3D bioengineered scaffold stained by Sirius red.

The collagen synthetic matrix is represented by network fibers stained in light red. This collagen matrix undergoes to a progressive degradation that was larger and more abundant in dynamic culture conditions. Furthermore, we observed new matrix deposition (darker red areas) within the hollow areas of the scaffold, but mainly at Day 16 in the scaffold cultured in perfusion.

4.5.3 3D microenvironment and hBM-MSC chondrogenic commitment: gene expression and Immunofluorescence assay

We next determined chondrogenic transcript expression within our scaffold and across the experimental timeline (**Figure 29a,b**). In the static condition, we observed marked downregulation of all transcripts tested (*SOX9*, *COL2A1*, *COL1A1*, *COL3A1*, and *ACAN*) at Day 8 and further

downregulation of all, except *SOX9*, and Day16. In contrast to above, the dynamic condition stimulated *COL2A1* upregulation (2-fold, $p < 0.05$) at Day 8 and further upregulation at Day 16 (5-fold, $p < 0.01$). This supported the hypothesis of chondrogenic commitment by the cooperative effect of growth factor and dynamic culture. Conversely, *COL1A1* (0.07-fold) and *COL3A1* (0.11-fold, $p < 0.05$), fibrocartilage tissue markers²²⁷, were both progressively downregulated across the timecourse. BM-MSC have an intrinsic tendency to differentiate towards an osteogenic lineage²⁰⁷ and the marked down regulation of *COL1A1* gene expression can be considered a further positive indication for commitment away from the default lineage.

SOX9 up-regulation was not observed in 3D perfused culture. This is consistent with previous reports, describing *SOX9* expression as an early marker of chondrogenesis in 3D culture driving regulation and activation of downstream targets including *COL2A1*^{228,229}. Further as an early marker of chondrogenesis, and consistent with previous reports, its upregulation may have occurred prior to day 8 followed by a return to basal levels and below thereafter^{230–232}. *ACAN* is reported to be a late marker²³³ and while downregulated at Day 8, we noted a subsequent significant upregulation from day 8 to day 16 in dynamic conditions.

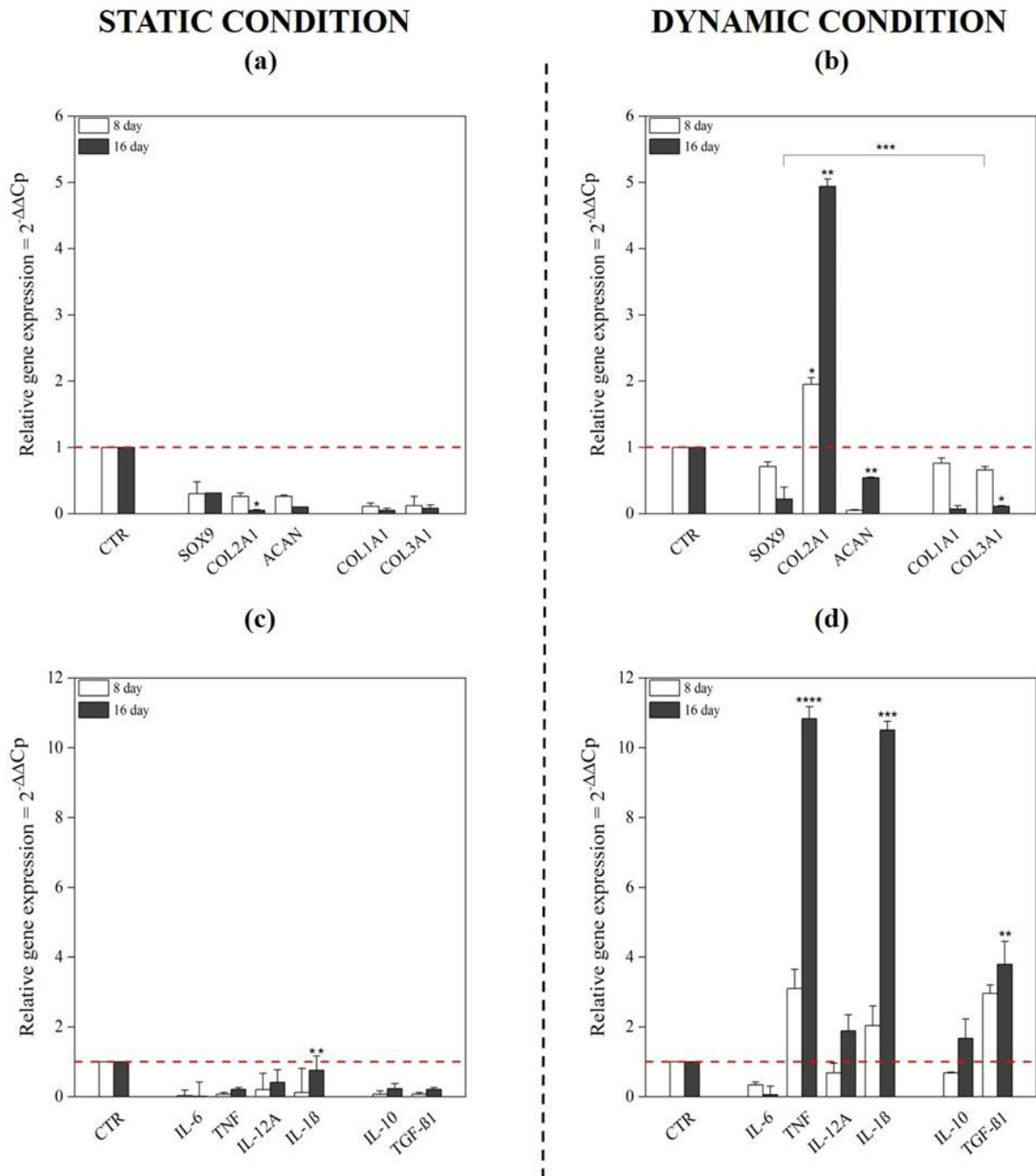


Figure 29 Gene expression profiling of chondrogenic markers and cytokines in static and dynamic culture conditions.

The mRNA expression levels of positive and negative chondrogenic markers (COL1A1, COL2A1, COL3A1, SOX9 and ACAN) and cytokines (IL-6, TNF, IL-12A, IL-1 β , IL-10 and TGF- β 1) were monitored using a RT-qPCR; untreated cells were used as control. In static condition, all investigated genes were down-regulated compared to the control (a, c); In contrast, COL2A1 mRNA levels showed, in dynamic culture, a significant up-regulation (4.9-fold) at Day 16 (b), as well as pro-inflammatory cytokines TNF and IL-1 β (10-fold) and anti-inflammatory ones as IL-10 and TGF- β 1 (2 and 3.8 fold changes) (d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (two-way ANOVA, $n = 3$).

Q-IF confirmed this trend, with increased type II collagen at day 16 in dynamic culture (0.4-fold, $p < 0.05$) and reduced type I and III collagen throughout ($p < 0.05$) (**Figure 30**). Day 8 displayed higher levels of collagen II signal, which significantly increased at Day 16 (0.4-fold, $p < 0.05$). Same investigation of collagen type I and III showed reduced signals at Day 16 compared to the control (see **Figure 30d**). This changed expression in collagen II and collagen III and I were indicative of an early commitment towards the specific phenotype ²¹⁷.

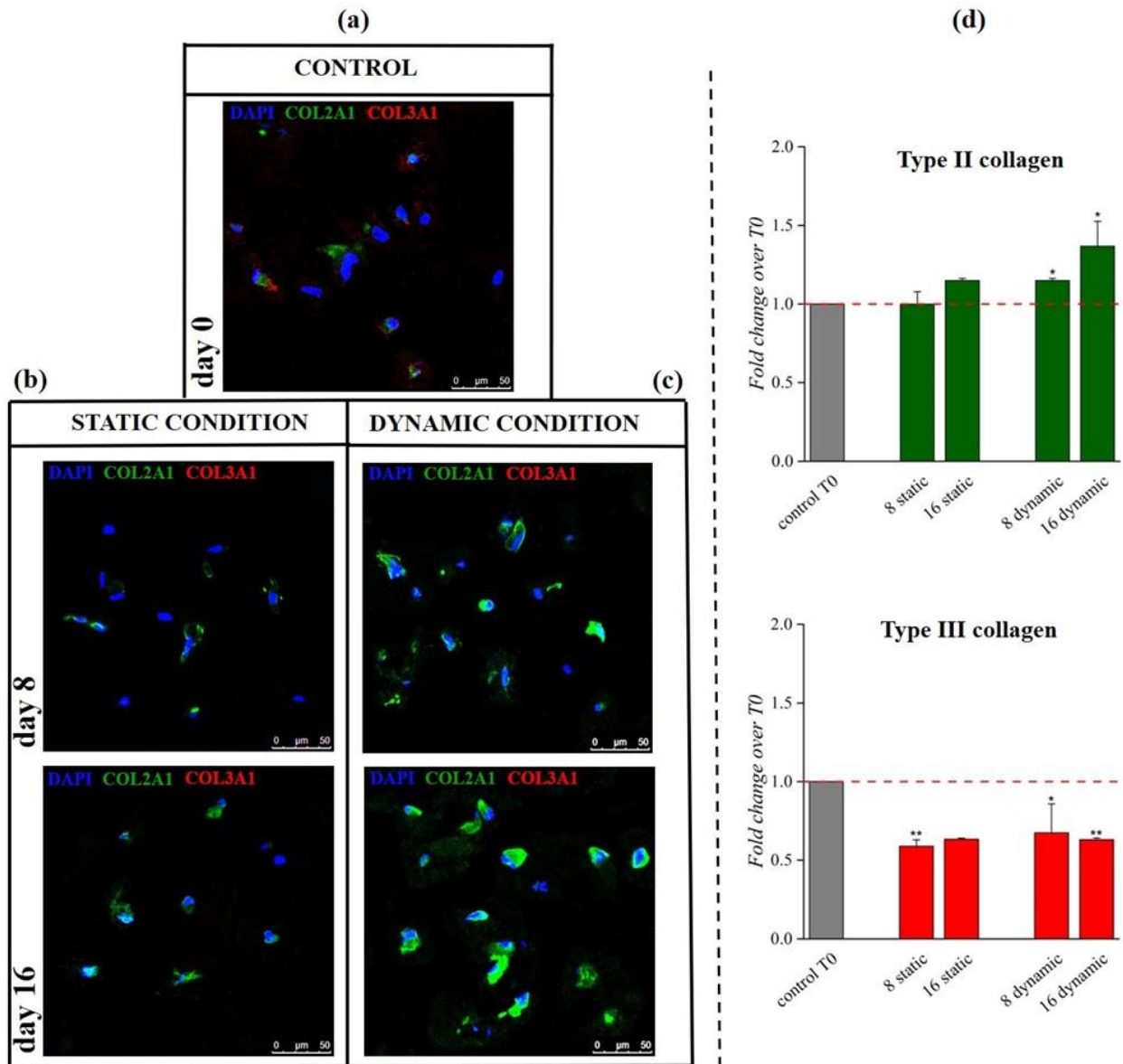


Figure 30 Immunofluorescence images and semi-quantitative analysis of type II and III collagen signals in both static and dynamic 3D culture.

IF assay at Day 8 and 16 of static (b) and dynamic (c) culture showed an increase of type II collagen signal (green) especially in dynamic culture at Day 16; type III collagen signal (red) was detected in the control sample at day 0 (a), but it has decreased in the other time points. Q-IF confirmed this trend, indicating a statistically significant increase of type II collagen protein (stained in green) at Day 16 in dynamic culture coupled with a significant reduction of type III collagen signal (stained in red) (d). Split channels and other IF data on type I collagen are reported in Supplementary Material. Scale bar: 50 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (one-way ANOVA, $n = 3$).

As a conceptual study to determine the suitability of scaffold to support chondrogenic differentiation and informative for future therapeutic applications, we sought to determine the immunomodulatory

reaction of cells to the synthetic environment by analysis of hBM-MSCs cytokine gene expression. Pro-inflammatory cytokines, TNF and IL-1 β displayed 10.8-fold and 10.5-fold upregulation ($p < 0.0001$) at Day 16 in dynamic culture (**Figure 29d**). This was coupled with elevated expression of anti-inflammatory IL-10 (1.67-fold, $p < 0.05$) and TGF- β 1 (3.80-fold, $p < 0.01$) at Day 16 (**Figure 29d**). On the other hand, no cytokines expression was monitored in static environment, suggesting that their overexpression was not induced by the 3D synthetic environment but more probably by the cell commitment (**Figure 29c**). These data may also suggest that the environment itself doesn't stimulate cytokine upregulation but that dynamic culture synergically influences cell commitment versus a specific phenotype and opens perspectives for the potential use of these biomaterials (both PLGA carriers and collagen) as injectable advanced therapeutical option for growth factor in situ delivery and local stem cells stimulation for cartilage tissue repair and healing.

4.5 Conclusion

Our presented 3D environment coupled to microcarrier-delivered hTGF- β 1 release and perfusion culture-driven enhanced mass exchange was effective in inducing chondrogenic commitment of hBM-MSCs. The versatility of our system enables future design for sustained release of multiple growth factors and/or immunomodulatory signals to further explore new solutions for *in vitro* cartilage repair and regeneration. Through the use of different MCs loaded with distinct biological signals a complex spatial-temporal delivery could be achieved. The described 3D microenvironment, therefore, opens concrete perspectives for the development of 3D bioengineered models to understand specific molecular and cellular composition of damaged systems.

Looking to clinical application, collagen hydrogels enriched with PLGA MCs have a relatively low dynamic viscosity (10^4 Pas)²²³ and body temperature (37°C) cross-linkage. This opens perspectives

for their future use as an intra-articular injectable advanced therapy for controlled-drug- delivery and/or factors for resident SC stimulation following *in vivo* administration.

**CHAPTER 5: Hybrid scaffold:
a novel strategy for improving mechanical properties**

5.1 Introduction

Articular cartilage injuries can be induced by traumatic events, as in the case of sports and occupational accidents, or be a consequence of autoimmune diseases (e.g., rheumatoid arthritis and gout)²³⁴. Unfortunately, current clinical management is restricted and is based mostly on palliative measures²³⁵ that are only intended to alleviate symptoms. Tissue engineering (TE) approaches, involving the association of different tools like cells, growth factors, and biomaterials could represent a concrete solution to these disorders^{89,95,236}. However, there are still some issues limiting their clinical application, such as the lack of biomaterials both with suitable mechanical properties as well as low susceptibility to enzymatic catabolism²³⁷. In this context, the synthesis of novel biomaterials to be used as three-dimensional scaffolds (3D) with suitable biomechanical features is extremely important to develop new surgical systems for *in vivo* implants^{238,239}. Typically, this is achieved using biodegradable and biocompatible materials, which may result from natural or synthetic sources²⁴⁰; furthermore, collagen-based scaffolds have been described as more appropriate to support chondrocytes or stem cells adhesion and proliferation, being more like the natural extracellular matrix (ECM) compared with other materials. In addition, they comply with all the relevant safety standards, being biocompatible²⁴¹. On the other hand, collagen is the most common structural protein of connective tissues in mammals and is involved in several biological functions, including support and nourishment²⁴². Currently, collagen intended for tissue engineering mainly comes from animal sources and exists in different physical forms, such as sponges, films, hydrogels²⁴³. However, this last one seems to be the most effective in ensuring homogeneous cells distribution. The only drawback is represented by low mechanical strength that is unsuitable for a possible *in vivo* implantation²⁴⁴. Another important consideration is that, despite there being many isoforms of collagens, only a few are used to produce biomaterials aimed at orthopaedic surgery. Type I collagen is currently the gold standard in the field of regenerative medicine²⁴⁵, indeed, it is preferred with respect to type II collagen owing to its simpler and quicker isolation, greater availability, as well as a

lower *in vivo* immunogenicity²⁴⁶. The common sources of collagen include equine tendons, bovine skin, and rat tails. A way to increase mechanical and enzymatic resistance of collagen-based scaffolds is to use a chemical crosslinking reaction. Glutaraldehyde and its derivatives are the most employed for this purpose⁶⁸. However, these reagents showed considerable toxicity on cells, which has discouraged their use²⁴⁷. An alternative strategy to improve mechanical and enzymatic properties consists in the development of hybrid scaffolds, obtained by the combination of different biomaterials. Recently, attention has focused on the possibility to incorporate different glycosaminoglycans (GAGs) in collagen membranes, thus obtaining biomimetic composite scaffolds with good porosity^{248,249}. Among GAGs, HA seems to be the most promising. From a chemical point of view, HA is an anionic linear non-sulfated polysaccharide of natural origin, commonly found both in the ECM of connective tissues and synovial fluid of joint, where it helps to reduce friction between bone cartilages³¹. A combination of GAGs and collagen was found more helpful and effective for cartilage regeneration²⁵⁰; furthermore, Mohammadi *et al.* have reported the preparation of hybrid scaffolds containing type I collagen derived from rat tail and hyaluronic acid (HA) at different ratios, confirming the better physical and chemical performance of blend scaffolds compared with single components²⁴⁸. Moreover, Tang and Spector have investigated the incorporation of different amounts of HA into type I collagen derived from bovine tendon and their findings demonstrated that HA can play a key role in enhancing the collagen mechanical behavior and *in vitro* chondrogenesis²⁵¹.

Previous studies have also highlighted that HA is able to induce chondrogenesis of human adipose-derived mesenchymal stem cells (hAD-MSCs) thanks to the interaction with CD44, a glycoprotein expressed on the cell surface acting as a receptor²⁵². To date, one of the major problems concerning the synthesis of hybrid scaffolds, especially those based on collagen/HA, is represented by the mixing of HA within the collagen solution. Although reactions in the homogeneous phase are more advantageous in terms of yield and control, they are very difficult to set up owing to the different stability between HA and collagen; indeed, while collagen shows good solubility and stability in

acidic aqueous solutions (e.g., acetic acid), the same acidic environment, especially if less than pH 4, can determine HA degradation, thus leading to a change in original molecular weight. The pH-induced degradation of HA is mainly ascribed to the cleavage of glycosidic bonds among monosaccharides ²⁵³. On the other hand, increasing hyaluronic acid molecular weight and concentrations leads to a rise in solution viscosity, which prevents its homogeneous incorporation ²⁵⁴. Another drawback is that in aqueous environments collagen and hyaluronic acid form polyion complexes (PIC), being respectively a cationic polypeptide and an anionic polysaccharide, thus forming scaffolds inhomogeneous in structure and with poor mechanical properties ²⁵⁵.

To overcome this, a potential solution is the incorporation process by heterogeneous phase ²⁵⁶; this approach could improve the integration of a greater amount of HA and guarantee the maintenance of the original porosity of collagen sponges. It should be noted that porosity is also required to ensure a correct exchange of oxygen and nutrients between cells and the surrounding environment.

5.2 Aims

In this study, a novel composite membrane scaffold formed by equine type I collagen and hyaluronic acid was here synthesized and characterized; FT-IR spectroscopy and thermal analysis were adopted to check the synthesis product; scaffold morphology and its mechanical properties were documented respectively by FE-SEM and tensile test. Finally, *in vitro* enzymatic degradation, as well as viability assay on human PBMCs and chondrocytes were performed to evaluate scaffold toxicity and its potential use as a biomaterial for tissue engineering.

5.3 Materials and methods

5.3.1 Materials

Type I equine collagen sponge was purchased from Euroresearch (Milan, Italy). Sodium hyaluronate (molecular weight 0.25-0.45 MDa) was purchased from Contipro Pharma, (Dolní Dobrouč, Czech Republic); Collagenase from *Clostridium histolyticum* was purchased from Sigma-Aldrich. All other chemicals were reagent grade from Sigma and Fluka. All chemicals were used without further purification unless otherwise specified.

5.3.2 Synthesis of the composite membrane

To synthesize composite membrane, here termed as HyCo, an equine type I collagen sponge (Euroresearch, Milan, Italy), in lyophilized form, was previously hydrated with a water solution of tetrabutylammonium salt of hyaluronic acid (HA-TBA). This reagent was synthesized using a cationic exchange process, starting from the sodium salt of HA, as better described elsewhere ²⁵⁷. In more detail, a plaque square-shaped with the size of 16 x 16 cm, with a thickness of 5 mm, and a weight of 2.0 grams was placed in contact with 100 mL of an aqueous solution, containing 1 % (w/v) of HA-TBA, until complete absorption. After that, the wet membrane was first frozen at a temperature of – 40 °C for 24 hours and subsequently lyophilized using a laboratory freeze dryer (ScanVac CoolSafe 55-9 Pro; LaboGene ApS, Lyngby, Denmark) to obtain the dry intermediate product. So, the resulting co-lyophilized membrane has been undergone a chemical crosslinking reaction (continuous boiling under reflux for 8 hours) in the heterogeneous phase using acetone as solvent, for activating terminal carboxyl groups of the hyaluronic acid (HA), as described detailing in the registered patent by Novagenit Srl (patent application n° 102018000005455). This reaction promoted the formation of amide and ester bonds among the carboxyl groups of HA and amine and hydroxyl groups of the type I collagen in order to entrap HA within the collagen matrix for avoiding its dissolution in aqueous

media. The crosslinked membrane has undergone a purification phase by washing with different solutions to ensure the removal of solvent residual. The cleansed membrane was finally freeze-dried, packed in medical paper, sterilized by beta irradiation (25 kGy) in a GLP-certified factory (STERIS S.p.A., Seriate BG, Italy), and stored at 4 °C temperature until its use.

A schematic representation of the whole process is shown in **Figure 31**. The percentage of HA into the final product was around 25% w/w and it has been derived gravimetrically, considering the difference in weight between the cross-linked membrane and starting collagen one.

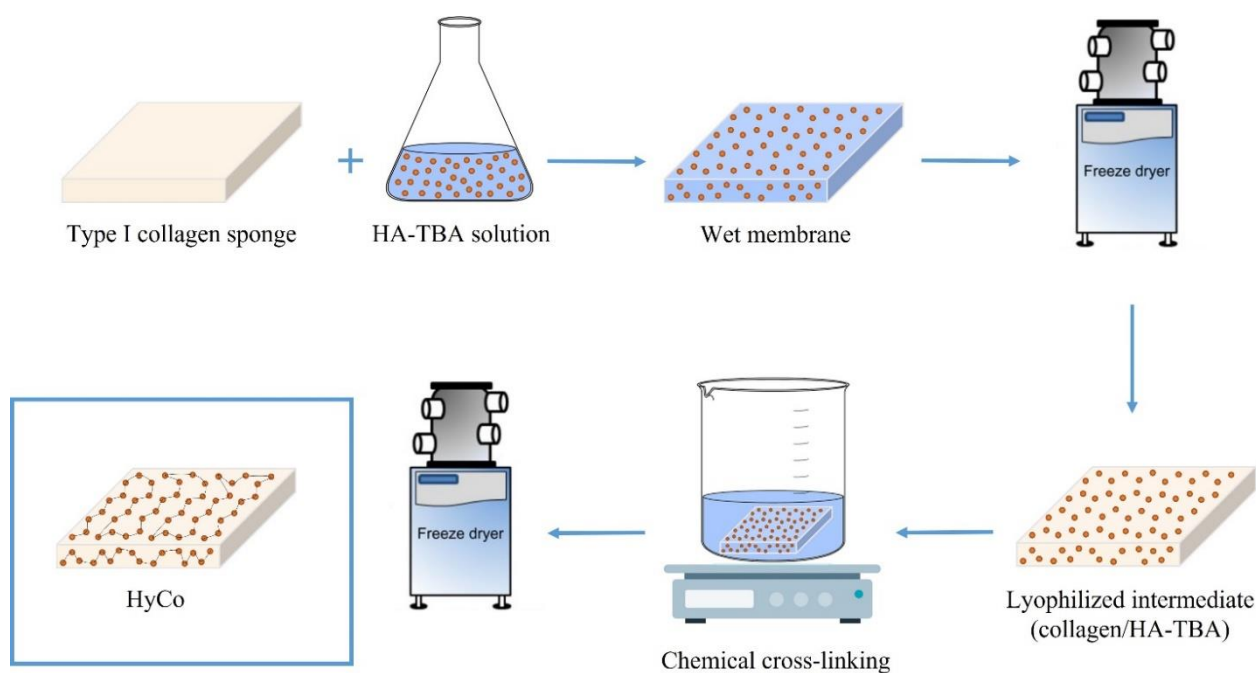


Figure 31 Schematic illustration of all process steps for synthesizing a composite scaffold.

A type I collagen sponge is hydrated by HA-TBA water solution and follow undergone to freeze-dryer for obtaining a co-lyophilized membrane. At this point, chemical cross-linking between collagen and HA is carried out through a reaction in heterogeneous phase. After chemical reticulation, the hybrid sponge is subjected to the final freeze-dryer that leads to HyCo.

5.3.3 FT-IR analysis

FT-IR analysis was performed with FT-IR Agilent Cary 630 Spectrometer (Agilent Technologies, Santa Clara, California, United States) equipped with an ATR diamond unit. In detail, roughly 100 mg of the dry sample of each membrane (HyCo and collagen alone) was tested in a wavenumbers

range from 650 to 4000 cm^{-1} (30 scans with a resolution of 4 cm^{-1} , 15 seconds per sample, and three replicates per sample) with the program MicroLab PC (Agilent Technologies, Santa Clara, CA, USA). The peaks corresponding to specific functional group were identified in final infrared spectra and then compared with those reported in the literature.

5.3.4 TGA and DSC analysis

Thermogravimetric analyses (TGA) were carried out in an air atmosphere with a Mettler Toledo TC-10 thermobalance from 25 °C to 700 °C at a heating rate of 10 °C/min. Differential scanning calorimetry (DSC) analyses were carried out in N_2 atmosphere with a Mettler Toledo DSC 822e from -50 °C to 160 °C at a heating rate of 10 °C/min.

5.3.5 Morphological characterization by Fe-SEM

Surfaces and sections morphology of scaffolds was observed by field emission-scanning electron microscopy (FE-SEM) (mod. LEO 1525; Carl Zeiss SMT OG, Oberkochen, Germany). Samples were first immersed in liquid nitrogen until freezing, and then fractured with a needle in order to expose their internal surface, placed on a double-sided adhesive carbon tape previously glued to an aluminum stub, and coated with a gold film (250 Å thickness) using a sputter coater (mod.108 Å; Agar Scientific, Stansted, UK), before observation.

5.3.6 Tensile test

Mechanical behaviors of all samples were measured in a tensile way at room temperature according to the guidelines established by the Standard Test Method for Tensile Properties of Polymer Matrix Composite Materials (ASTM D3039/A3039M) using an Instron 5967 machine with Bluehill software

(Norwood, MA, USA) equipped with a 10 N load cell. Samples were shaped to obtain specimens having a gauge length (L_0) of 22 mm and a width (W) of 10 mm; sample thickness (S) was ranging from 3 to 5 mm. A monoaxial deformation was applied to the sample with a speed of 10 mm/min, and force (F) and elongation (L) during traction was registered. The value of force (F) provided by the instrument was divided by the sample area ($A = W \times S$) to obtain the strength values ($\sigma = F/A$). The deformation values (L) during the run were compared to the initial length to obtain values of strain ($\varepsilon = (L - L_0)/L_0$); the ultimate tensile strength ($\sigma_{\max}$, expressed in KPa) was calculated as load to failure/cross sectional area of the sample.

5.3.7 Absorbency and swelling ratio assays

Each membrane was cut (Swann-Morton®, Sheffield, England) to obtain three samples with dimensions of 5 x 5 cm and a thickness of 3 mm (HyCo) and 4 mm (collagen). Samples were weighted by an analytical balance (Model LE 324S-Sartorius, Gottingen, Germany) and their dry weights registered; moreover, the volume (V) of each sample was calculated, considering their initial length, width, and thickness. Each membrane was placed in a Petri dish and hydrated with a volume of preheated (37 °C) saline aqueous solution (containing 142 mM of NaCl and 2.5 mM of CaCl₂) equivalent to 40 times the sample's mass. At this stage, all samples were transferred in a standard incubator (37 °C, 5% carbon dioxide (CO₂), and 85% relative humidity (RH)) for mimicking physiological conditions. The difference in mass, before and after the hydration phase, was registered and the resulting absorption capability (mg/mm³) was calculated using the equation below (1). Absorbency was measured at two time intervals: 30 minutes and 24 hours.

$$\text{Absorbency (mg/mm}^3\text{)} = \frac{W(\text{wet}) - W(\text{dry})}{V} \quad (1)$$

Where W(wet) is the weight of the sample after different hydration times while W(dry) is starting raw weight, and finally V is the initial volume of the respective specimen. At every scheduled time point, the matching samples were collected and suspended by one corner with tweezers for 30 seconds, in order to remove the excess solution by gravity and finally carefully reweighed ²⁵⁸.

Moreover, swelling behavior of different membranes in phosphate-buffered saline (PBS) solution (pH 7.4) at 37 °C for 24 hours was monitored. To achieve that, lyophilized samples were weighted and then placed in PBS at 37 °C. The swelling ratio (%) was calculated using the following equation (2)

$$\text{Swelling ratio (\%)} = \frac{W(\text{wet}) - W(\text{dry})}{W(\text{dry})} \times 100 \quad (2)$$

Where W(wet) is the weight of the sample after different hydration times and W(dry) is starting raw weight. All assays were carried out in triplicate and for each, the mean value and the standard deviation were reported.

5.3.8 In vitro enzymatic degradation assay

The *in vitro* degradation of the two membranes was performed using a bacterial type I collagenase from *Clostridium histolyticum*, according to a protocol described elsewhere ²⁵⁹. Samples (about 40 mg) were accurately weighed (W_0), hydrated with 4 mL of 0.1 M Tris–HCl (pH=7.4), containing 5 mM CaCl₂ and 50 mg/L NaN₃ and then placed at 37 °C. After one hour of incubation, 4 mL of collagenase solution (2.4 U/mL) in Tris–HCl (pH 7.4, 37 °C) were added, to give a final collagenase

concentration of 1.2 U/mL. Degradation was evaluated at desired time intervals by stopping collagenase activity with the addition of 1 mL of 0.25 M Na₂-EDTA to each sample. A gravimetical method was used to determine the weight-loss of the samples: residual samples were washed three times against distilled water before freeze-drying and weighed again (W_t). The extent of degradation was expressed as percentage of the original weight of the samples (**see equation 3**) and reported in function of the degradation time. All tests were performed in triplicate and standard deviations were reported

$$\text{Weight (\%)} = \frac{\text{Weight time-point}}{\text{Weight time-zero}} \times 100 \quad (3)$$

5.3.9 Cells harvesting and cytotoxicity assay

hPBMCs were obtained from Buffy Coats donated from University Hospital Blood Bank, as anonymous material from healthy donors and separated by Ficoll-Hypaque gradient density (Sigma-Aldrich) following standard techniques¹⁷⁴. After isolation, cells were re-suspended in RPMI medium, supplemented with 10% heat-inactivated FBS, 10 mg/mL L-glutamine, and penicillin/streptomycin (50 U/mL). Cells were then seeded in 12-well plates at a density of 1×10^6 cells/mL and incubated in a humidified atmosphere containing 5% CO₂ and 95% air. Cell metabolic activity was analyzed using the MTT assay. Briefly, cells were incubated along with membranes (concentration about 6.7 mg/mL) for 24 and 48h using porous transwells (3 μ m) (Greiner bio-one, No. 665631) in order to maintain always separated the cells (in suspension) from membranes. Adopting this protocol, the cells and membranes are still in contact through the culture medium but the cell migration and their resulting diffusion within scaffold pores are prevented. Following treatment, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added (1 mg/mL) to each well and incubated at 37°C for

an additional 4 h. Plates were centrifuged at 300 g for 10 min and the supernatants were completely removed. Formazan products were dissolved in dimethyl sulfoxide (DMSO). Absorbance was determined at 570 nm using a microplate reader (Infinite F200 PRO, Tecan Group Ltd., SW). All assays were performed in triplicate (N=3). For MTT assay on hPBMCs, three independent experiments were performed in triplicate, each one on a single subject (N=3). Cell metabolic activity, a surrogate of viability, was calculated as the percentage of control group, considered as 100%. Percentage viability of cells was calculated according to follow equation (4):

$$\text{Cells metabolic activity (\%)} = \frac{\text{Abs of sample} - \text{Abs of blank}}{\text{Abs of control} - \text{Abs of blank}} \times 100 \quad (4)$$

Human chondrocyte line (Cell Applications, Inc. Lot 2672) was cultured on HyCo membrane for 28 days and cell viability was determined by an adenosine-5'-triphosphate (ATP) assay with the Promega CellTiter-Glo® 3D Cell Viability kit (Promega Italia S.r.l., Milan, Italy). Cylindrical shape scaffolds with a diameter of 6 mm and height of 4 mm (volume = 113 mm³) were obtained through a sterile biopsy punch (B Life S.r.l., Treviso, Italy), and seeded with 110 µl of chondrocyte suspension at a density of 1×10⁶ cells/mL. Relative Luminescence units (RLUs) were recorded with a LuMate® luminometer (Awareness Technology Inc., Florida, USA) and resulting data were analyzed through LuMate Manager software.

5.3.10 Statistical analysis

Comparison between two independent groups two-tailed independent Student's T test was performed. P values <0.05 were accepted as significant.

5.4 Results

The development of a novel composite material (we termed as HyCo) combining equine type I collagen and hyaluronic acid was here investigated.

For this purpose, a lyophilized sponge, composed of type I native heterologous collagen extracted from the equine flexor tendon, was previously hydrated with a water solution of hyaluronic acid and subsequently chemically crosslinked in order to improve their mechanical properties as well as endurance to enzymatic degradation. Chemical and physical characterization was carried out in order to optimize synthesis protocol; a commercial collagen scaffold was used as reference. Chemical bonds' nature and differences in terms of functional groups between pure collagen and the novel crosslinked scaffold were investigated by FT-IR spectroscopy and the resulting spectra of all samples are shown in **Figure 32a**. Specifically, the spectrum of pure collagen showed traditional absorption bands of proteins, which are characterized by a predominant peak at a wavenumber about 1634 cm^{-1} for amide I (C=O stretching), followed by the presence of other two peaks at wavenumbers of 1539 and 1235 cm^{-1} , associated with amide II (N-H deformation) and amide III (tertiary C-N stretching vibration)²⁶⁰ respectively as clearly shown **Figure 32b**. Moreover, in the spectrum of un-crosslinked type I collagen, two other amide bands were evident at frequencies of 3289 cm^{-1} (amide A; N-H stretching) and 2931 cm^{-1} (amide B; C=O stretching). In the case of the HyCo scaffold, a new peak in correspondence of the wavenumber of 1735 cm^{-1} appeared. Furthermore, all bands associated with amides were decreased in intensity after crosslink reaction.

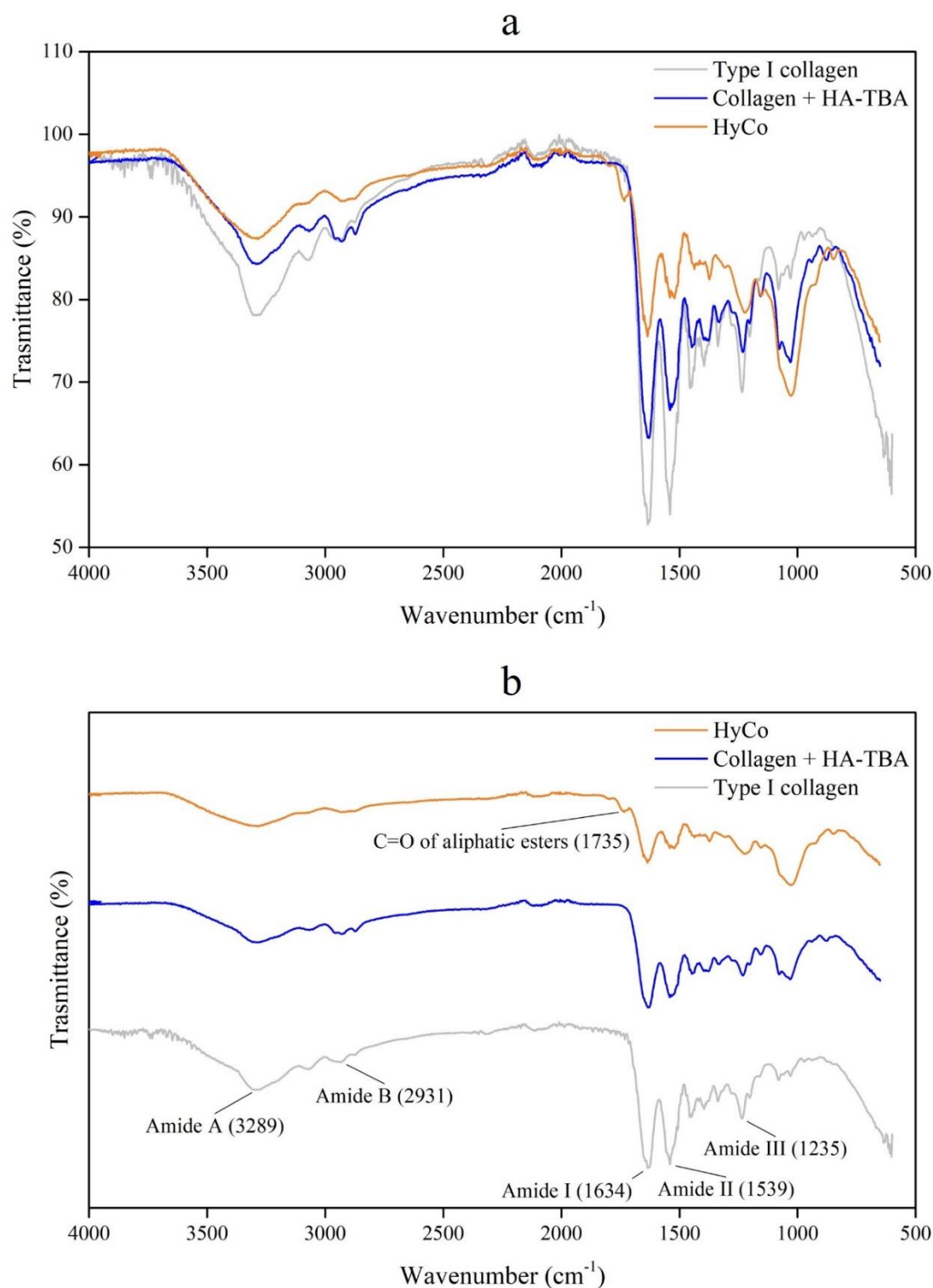


Figure 32 FT-IR spectra of equine type I collagen, co-lyophilized intermediate (collagen/HA-TBA), and final HyCo membrane.

All spectra were acquired at room temperature, using wavenumbers ranging from 650 to 4000 cm^{-1} , with a resolution of 1 cm^{-1} . The resulting merge of all acquired spectra is shown in the upper graphic (**a**), while split signals of different samples are reported below (**b**). The representative peaks of major functional groups were assigned.

5.4.1 Thermal behavior

Both thermal gravimetric (TGA) and Differential Scanning Calorimeter (DSC) analyses were also performed. **Figure 33a-b** shows findings of them for type I collagen and HyCo membrane. From TGA analysis, we obtained the curves associated with the mass loss (%) of samples during the warming process up to 700 °C. TGA curves of pure collagen and HyCo showed a similar profile with a stage of losing weight ranging from room temperature to 200 °C due to the evaporation of physisorbed water (**Figure 33a**). By analyzing curves' trend, both scaffolds have hygroscopic properties. A further weight loss was shown between 250 and 550 °C related to the decomposition of collagen structure followed by finally slight loss between 550 and 700 °C resulting from combustion of residual organic components ²⁶¹. Furthermore, DSC data confirmed that HyCo had different thermal behavior as shown in **Figure 33b**, where collagen as such and HyCo exhibited different endothermic peaks at 97.15 and 102.32 °C, respectively. The denaturation temperature of HyCo was higher than that of the pure collagen. This suggests that the thermal stability of the collagen was enhanced by the presence of cross-linked hyaluronic acid. Therefore, the reaction can be also considered successful because HyCo showed a unique and uniform DSC curve shape that was also used for quality control of cross-linked scaffolds.

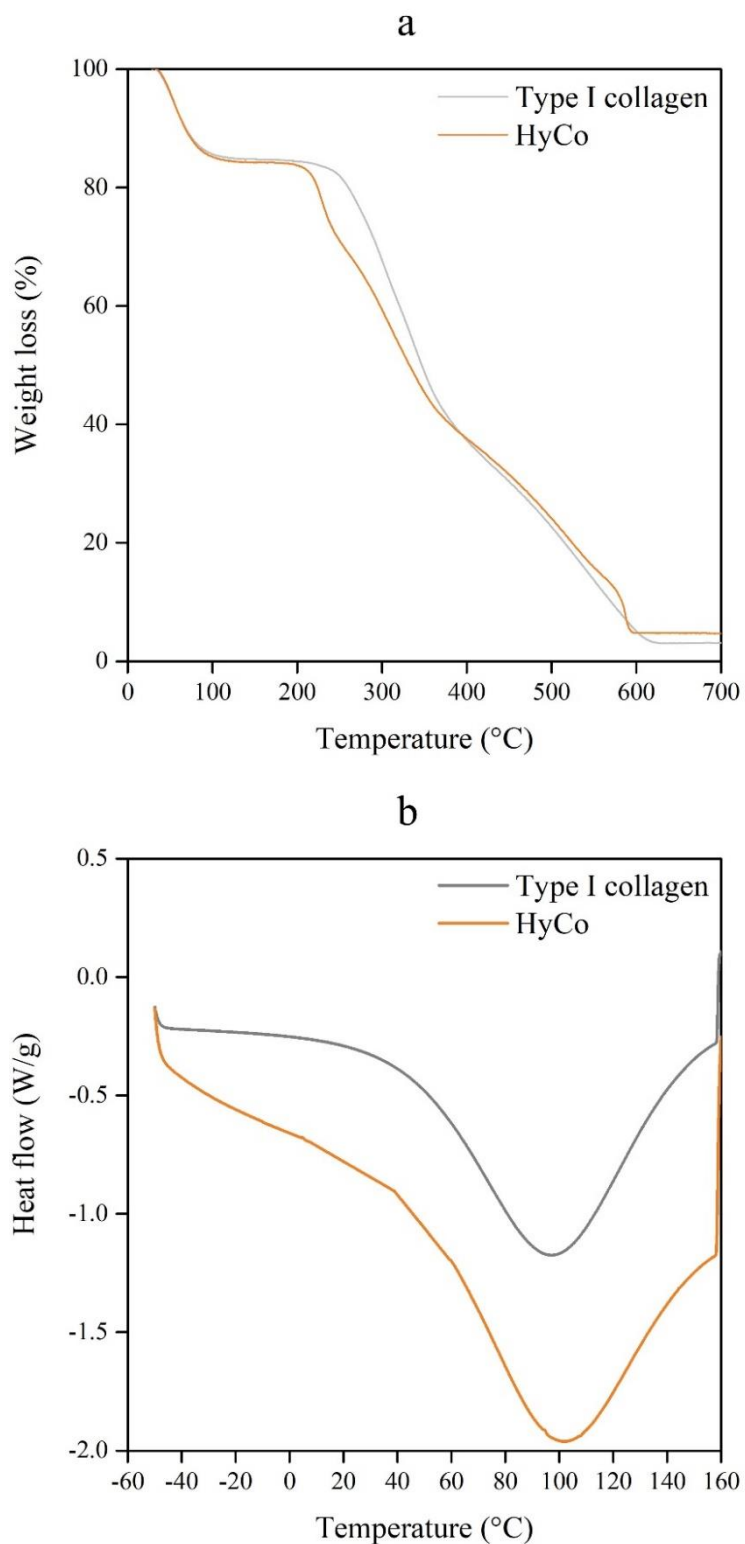


Figure 33 TGA and DSC analyses of pure collagen and composite scaffold.

TGA thermograms within the warming range from 0 to 700°C (**a**); DSC profiles were recorded using a calorimeter with samples shielded in aluminum containers in the temperature range of -50°C and 160°C in nitrogen atmosphere. The DSC scan shows an endothermic peak at 100°C attributed to water loss/evaporation (**b**)

5.4.2 Morphological analysis by FE-SEM

Samples were further characterized by field emission electron scanning microscopy to obtain information about their morphology and surface porosity. SEM images of the cross-sections of both scaffolds, obtained after cryo-sectioning showed a highly porous structure (see **Figure 34a-d**) with a continuous network of open pores. In the case of collagen scaffold, a dense random orienting fibers network, very slim in thickness was observed, especially on the surface (**Figure 34e**). In contrast, the cross-linking process with HA has changed the morphology and the porosity of the scaffold, leading to a considerable thickening of the fibers as shown in **Figure 34f**. Finally, from digital analysis of SEM images, superficial pores of the sample after chemical reticulation had a round or oval shape with an average diameter about of $150\pm70\text{ }\mu\text{m}$.

Type I Collagen

HyCo

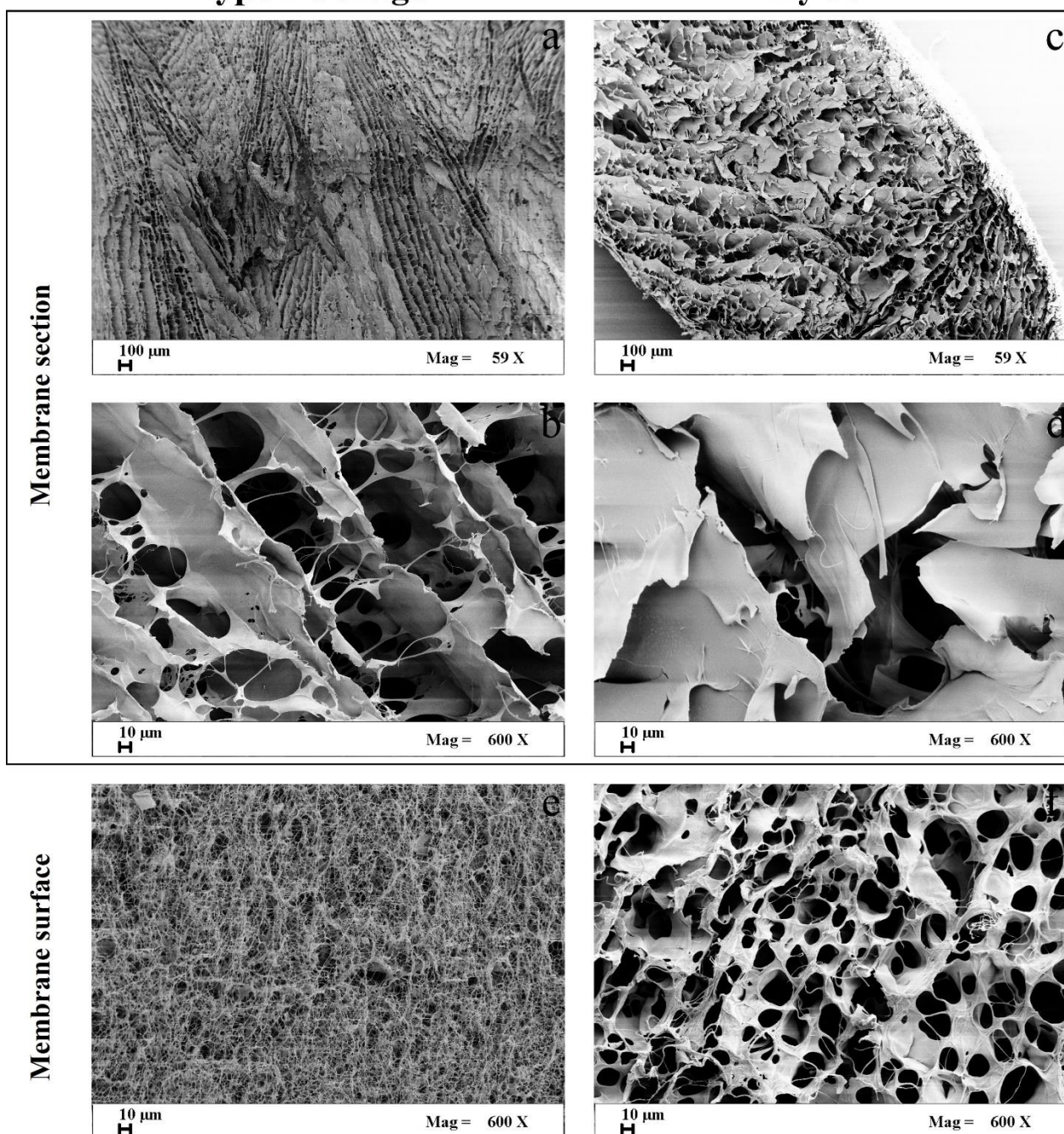


Figure 34 Comparison between SEM images of type I collagen membrane and HyCo one.

From micrographs is clearly a different porosity and internal organization of two membranes; however, both showed interconnected voids

5.4.3 Mechanical characterization

To evaluate differences in terms of mechanical behavior following cross-linking protocol, tensile tests in wet conditions (100% humidity) on pure and cross-linked collagen scaffolds were performed. In more detail, when mechanical properties were evaluated, all scaffolds exhibited a similar stress-strain trend (**Figure 35a,b**). Specifically, the curves showed three distinct zones: a first linear region, termed Toe, in which stretching out of collagen fibers occurs in response to low mechanically loading. This zone ends under 10% of strain until all curled fibers straighten and it is followed by a second linear region, in which the collagen fibers respond linearly to a higher load. Finally, the last stretch is associated with irreversible failure. In the first linear region (Toe), Type I collagen membranes presented a Young Modulus of 0.06 ± 0.04 kPa (see **Table 6**), while in the second linear one Young Modulus reached a value of 1.57 ± 0.08 kPa. At the breaking point, the elongation and tensile strength were respectively 54.04 ± 8.20 and 36.91 ± 0.24 . On the contrary, HyCo displayed an increase of both Young modulus with respect to just collagen, achieving a value of 2.17 ± 1.35 ($p \leq 0.05$) and 9.96 ± 0.53 kPa ($p \leq 0.001$) respectively for the first linear region and the second one. In this last case, the percentage of elongation at break was notably decreased to 12.09 ± 1.16 whilst the resulting tensile strength raised to 97.36 ± 3.58 .

Table 6 Young's modulus, stress at break, and elongation at break of HyCo scaffold compared with commercial type I collagen membrane.

	Type I collagen	HyCo
Humidity (%)	100	100
Young's modulus of TOE region (KPa)	0.06 ± 0.04	2.17 ± 1.35
Young's modulus of linear region (KPa)	1.57 ± 0.08	9.96 ± 0.53
Elongation at break (%)	54.04 ± 8.20	12.09 ± 1.16
Tensile strength at break (KPa)	36.91 ± 0.24	97.36 ± 3.58

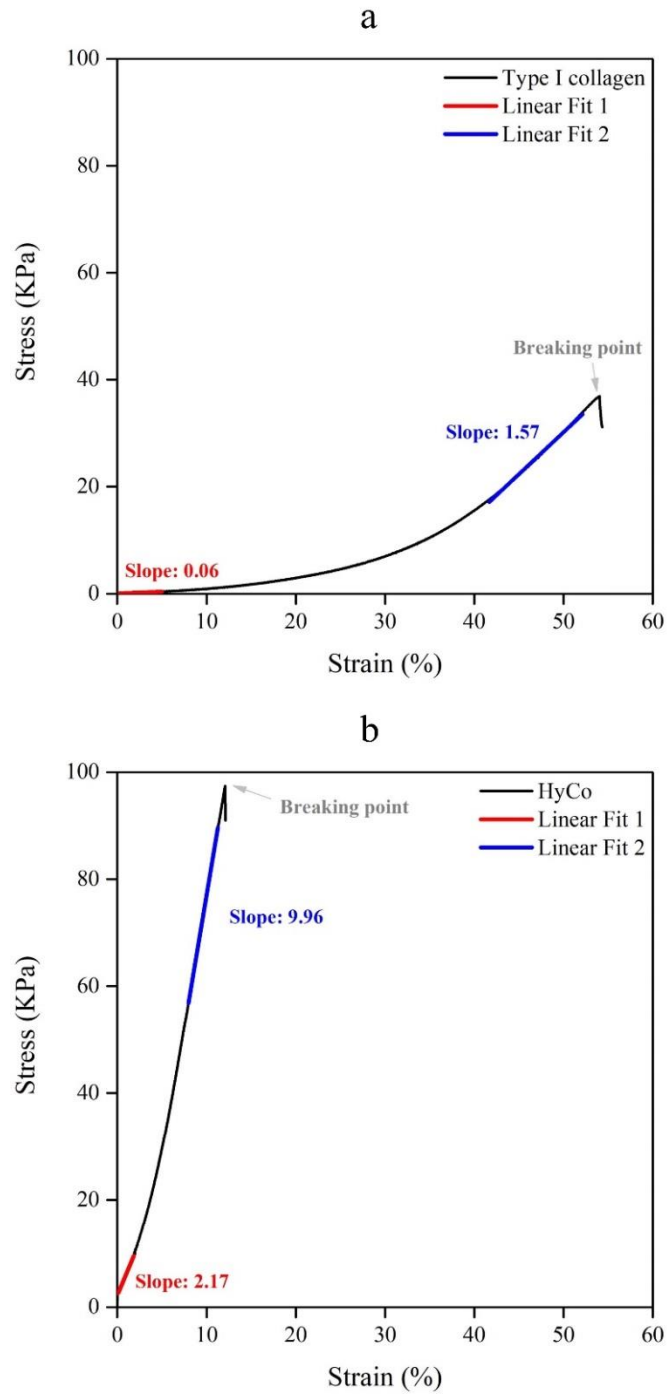


Figure 35 Stress-strain curves resulting from tensile tests of type I collagen and HyCo and related Young's Moduli.

Tensile tests were carried out both on type I collagen for control purposes (**a**) and on cross-linked membrane HyCo (**b**).

5.4.4 Absorbency and swelling behavior

HyCo and pure collagen membranes were tested to evaluate their ability in absorbing and retaining over time a saline solution at body temperature, according to a protocol described in the British Pharmacopoeia (1995) for textiles aimed at medical purposes. Absorptive capacities after 30 minutes and 24 hours of incubation at 37 °C are shown in **Figure 36a**. From comparison between the two kinds of scaffolds, absorption capability of the hybrid one was higher compared to collagen alone, as confirmed by absorbency values both after 30 minutes and 24 hours. Type I collagen was the least hydrated membrane, absorbing 0.52 ± 0.04 mg/mm³ and 0.44 ± 0.04 mg/mm³ of saline solution after 30 minutes and 24 hours respectively. Conversely, HyCo membranes showed the highest values, reaching 1.25 ± 0.04 mg/mm³ after 30 minutes and 1.33 ± 0.01 (p ≤ 0.0001) mg/mm³ after 24 hours. In this study, the swelling ratios (%) in PBS (pH 7.4) related to both membranes were also determined, and the findings are shown in **Figure 36b**. Type I collagen exhibited a rapid absorption capability, achieving the highest value of swelling ratio $2,848 \pm 266$ % after 1 hour. However, this parameter tended to markedly decrease over time, reaching a percentage of 2550 ± 222 % following 24 hours. Differently, HyCo achieved the highest swelling ratio at 5 hours with a percentage of 1842 ± 143 % (p ≤ 0.05), showing a more rising and reproducible trend during 24 hours, as confirmed by the low standard deviations. Following hydration phase, collagen tended to lose its original shape, thus reducing its size over time, as shown in **Figure 36c**. Conversely, HyCo seemed to maintain its native structure, demonstrating a good shape memory during the whole incubation period.

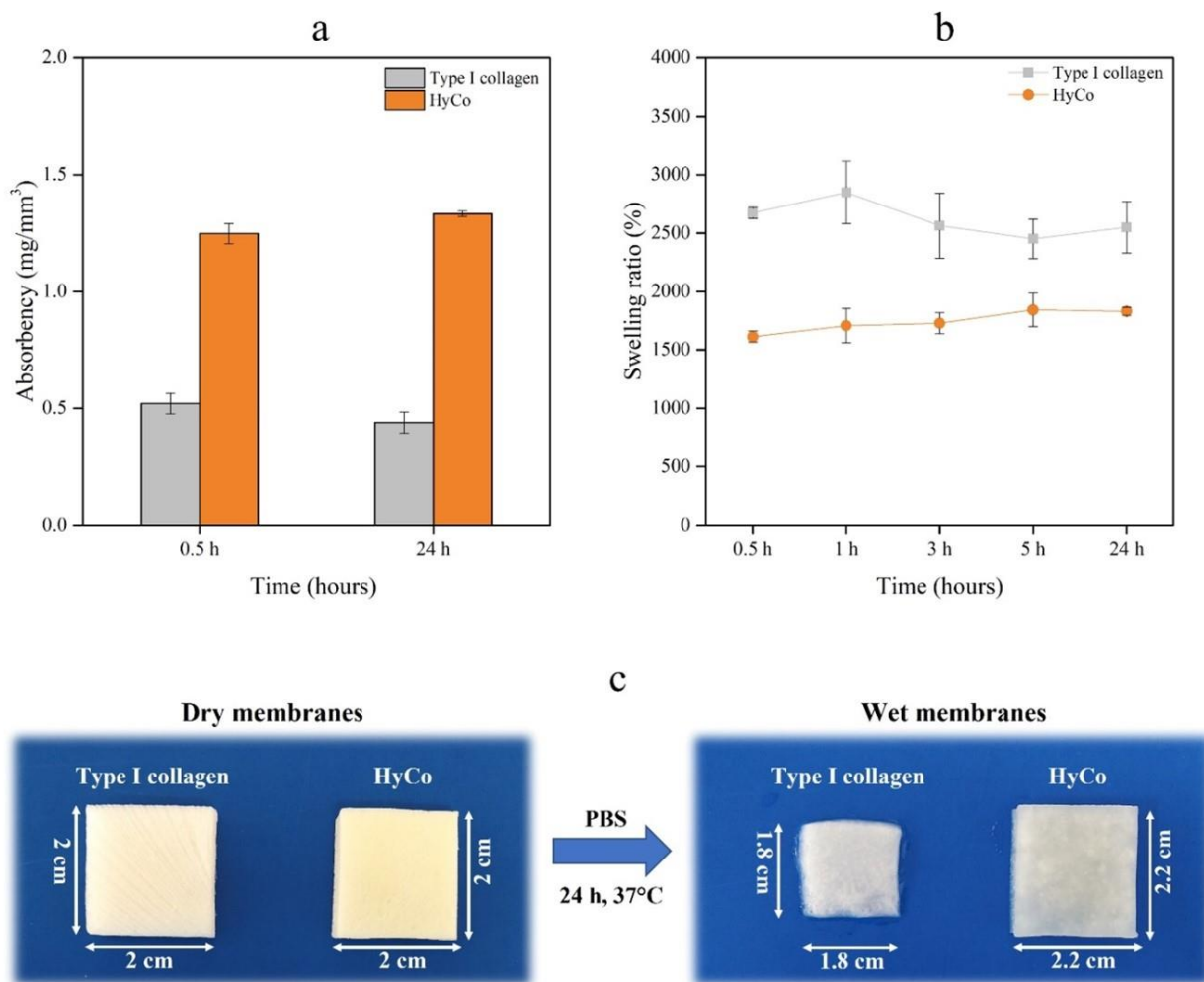


Figure 36 Absorbency and swelling ratio assays on pure type I collagen and HyCo membrane.

Absorption capability of a saline solution (142 mM NaCl, 2.5mM CaCl₂) by type I collagen and HyCo at 30 minutes and 24 hours expressed as mg/mm³ (a) and related swelling ratio (%) at different time points (b). Data are presented as mean $\pm$ standard error. Pictures of dry samples and following 24 hours of incubation with PBS at 37°C (c).

5.4.5 Enzymatic degradation and cytotoxicity assay

To evaluate kinetic profiles, *in vitro* enzymatic degradation test was performed, using a type I collagenase derived from *Clostridium histolyticum*. Degradation behavior in the presence of collagenase at 37 °C (pH 7.4, 1.2 U/mL) is reported in **Figure 37a**. Results indicated that uncross-linked collagen was quickly degraded, losing 71 $\pm$ 4.5 % of its mass in just 24 hours; whilst the cross-linked one has lost 4.1 $\pm$ 1.6 % of its weight after the same time, demonstrating a higher resistance to enzymatic activity. After 72 hours of incubation with collagenase solution, the not cross-linked

scaffold has been completely degraded. In contrast, scaffold HyCo showed a final degradation rate of 31.64 ± 3.5 %.

Furthermore, cytotoxicity effects of scaffolds were explored on hPBMCs isolated from healthy donors to provide preliminary safety evidence directly on human primary cells. Cells were incubated for 24 and 48h with membranes weighing 10 mg (final concentration within each well about 6.7 mg/mL), and their metabolic activity was analyzed by MTT assay (**Figure 37b**). Results demonstrated that despite high concentration tested, uncross-linked collagen has a very mild cytotoxic effect on hPBMCs, showing metabolic activity of 86.93 ± 2.8 % and 67.81 ± 2.02 % ($p \leq 0.01$), respectively after 24 and 48 hours. In contrast, HyCo has shown a reduction of this parameter, achieving 81.92 ± 1.9 % following 1 day and 76.37 ± 1.2 % ($p \leq 0.001$) after 48 h.

Human chondrocytes were also cultured on HyCo membranes for 28 days and their cell viability was monitored by an adenosine-5'-triphosphate (ATP) assay. From experimental data, it can be observed a significant statistically increase in cell viability after 14 and 21 days of culture compared to control (cells just seeded), as documented in **Figure 37c**.

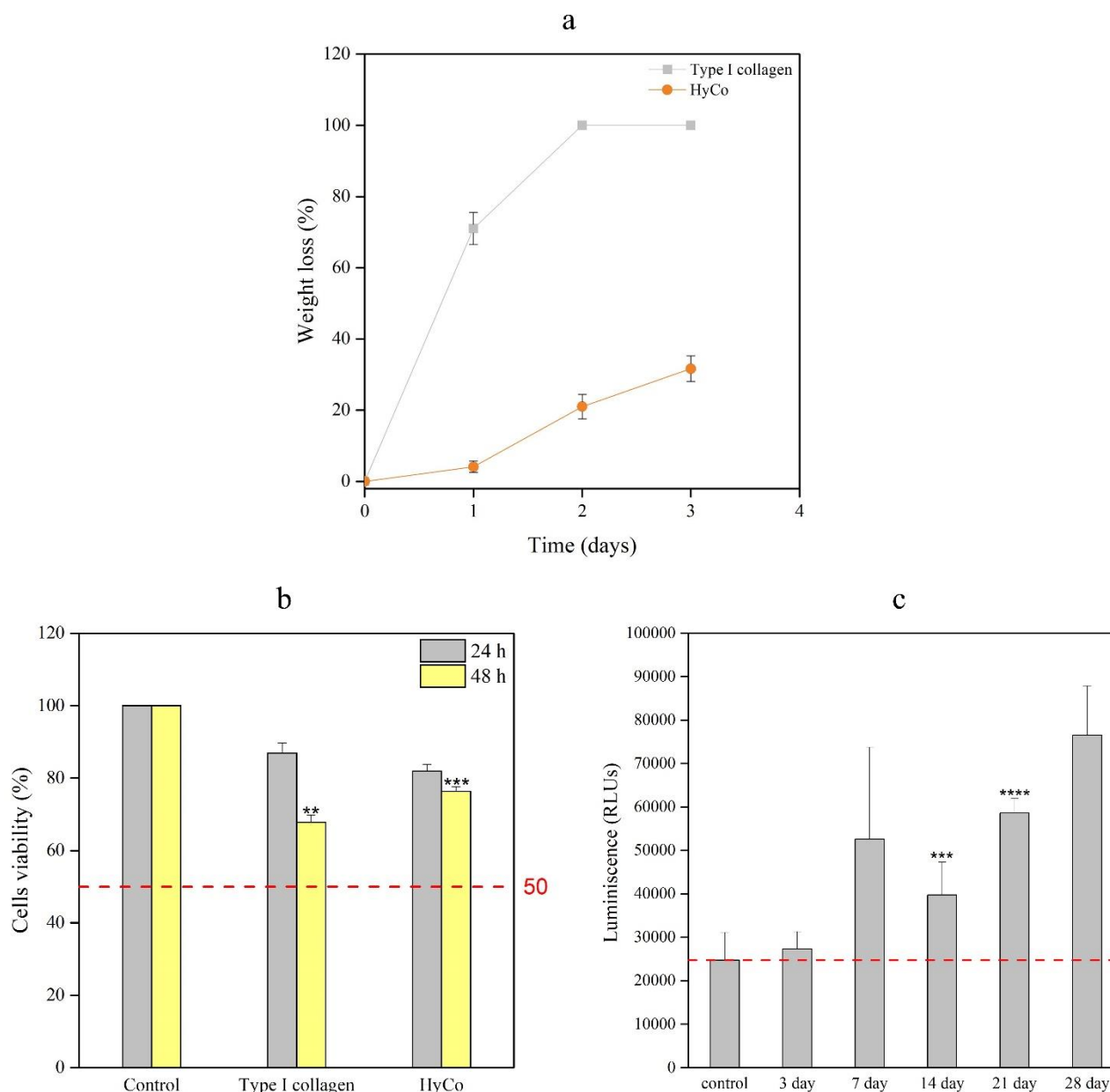


Figure 37 Degradation profiles of type I collagen and HyCo membrane with related cytotoxicity on human PBMCs and chondrocytes.

Comparison between enzymatic degradation of equine type I collagen and HyCo membranes following treatment with collagenase from *Clostridium histolyticum*, 1.2 U/mL in Tris-HCl pH 7.4, CaCl₂ 2.5 mM, NaN₃ 25 mg/L, T 37 °C (a); Scaffolds cytotoxicity was evaluated on human peripheral blood mononuclear cells (hPBMCs) by MTT assay. The histograms report the mean percentage of viable cells compared to controls (untreated cells, 100%) (b). Human chondrocytes within HyCo scaffolds were cultured for 28 days and their ATP content was measured. The histograms report the mean of relative luminescence units compared to control (c). The experiments were analyzed by two-tailed Student's t-test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p \leq 0.0001$; $n = 3$

5.5 Discussion

The rise of cartilage tissue engineering has been hampered by the intrinsic trouble in reproducing a stable hyaline-like cartilage tissue. The reasons are not only associated with difficulty to induce and maintain over time the chondrogenic phenotype of stem cells or chondrocytes^{262,263} but also with the lack of biomaterials with suitable mechanical properties for *in vivo* application²⁶⁴. Current management of cartilage lesions often requires invasive surgical procedures²⁶⁵, which could be enhanced in terms of outcomes and prognosis by the development of advanced scaffolds²⁶⁶. In the context of tissue engineering, type I collagen is one of the most employed natural proteins as a scaffold, owing to its *in vivo* biodegradability and full safety as well as low antigenic potential²⁶⁷. Moreover, despite this protein may be extracted from different animal sources, including bovines and porcine, equine one is the most used since is almost free from zoonosis risk²⁶⁸, and for this reason, we have selected it for our experimentation. However, from a clinical point of view, this material presents several limitations including high susceptibility to enzymatic degradation by collagenases, structural instability following hydration, and poor mechanical resistance; all these drawbacks decreased successful clinical outcomes significantly, thus discouraging its application.

Departing from these weaknesses, we described here a novel process for synthesizing hybrid scaffolds, combining hyaluronic acid with equine type I collagen to obtain a composite material with improved physico-chemical and biomechanical properties. The choice of the two biopolymers for HyCo synthesis was due to the specific application proposed; indeed, it is well-known that cartilage is formed by collagen and proteoglycans. Proteoglycans are macromolecules composed of a protein backbone (core) to which long chains of glycosaminoglycans (GAGs) are covalently linked; whereas, GAGs can be sulfated such as chondroitin and keratan or non-sulfated, as in the case of hyaluronic acid. This last component has the ability of both retaining a high amount of water and of interacting with different proteoglycans, as a coupling agent for the assembly of large aggregates, which provided the greater mechanical strength to cartilage tissues. In pathological conditions, collagen fibrils are

degraded, and proteoglycans aggregates are cleaved from the hyaluronic acid backbone, leading to the deterioration of the physiological network with the loss of its functionality ²⁶⁹. Therefore, intending to mimic and restore the natural environment where chondrocytes reside, we decided to set up a novel hybrid scaffold using the combination of the two principal components of which cartilage ECM is composed.

So that, HyCo properties and its biomimetic structure could be a potential adjuvant for treating cartilage diseases; however, as already reported in the introduction, one of the major problems to tackle in synthesizing hybrid scaffolds based on hyaluronic acid and collagen is represented by the association of these components. As is widely known, they have different behavior in terms of solubility and in aqueous solution tend to generate polyion complexes, which interfere with proper scaffold structuring ²⁵⁵. To overcome this, an incorporation process by heterogeneous phase was developed, starting from a collagen sponge and a hyaluronic acid water solution. In other words, the collagen sponge was first soaked with HA solution and then freeze-drying. The resulting copolyophilized sponge then underwent a chemical cross-linking through activation of carboxylic functions to entrap HA within the collagen matrix (**Figure 31**), thus avoiding its early dissolution in contact with biological fluids ²⁷⁰. It should be noted that the development of scaffolds by freeze-drying processes is very advantageous in the pharmaceutical field because water is the only medium employed as porogen before freezing ²⁷¹. Moreover, this process gives a highly porous structure to materials, boosting their hydrophilicity. This is a very important aspect to consider since all materials designed as scaffolds for use in cellular therapies must be extremely porous to accommodate cells growth and proliferation. Furthermore, the porosity impacts also on matrix permeability, required for exchanges of nutrients or waste products between the scaffold and external environment ²⁷².

In this study, the process to obtain scaffolds with a percentage of HA around 25 % in weight was optimized. Furthermore, HA percentage can be also changed by operating on its starting concentration

in solution during hydration phase of the sponge; this procedure makes the process extremely advantageous in terms of versatility. However, it should be noted that when using high concentrated HA solution (> 15 mg/mL) the higher viscosity of the liquid hampers the proper solution absorption into collagen sponge; therefore, higher percentages of HA loading are not possible. Chemical characterization by FT-IR analysis suggested that cross-linking leads mainly to an esterification reaction as confirmed by the presence of a new peak associated with carbonyl stretch of aliphatic esters within FT-IR spectrum related to HyCo (**Figure 32b**). Therefore, we hypothesized that HA could form ester-type bonds between its carboxylic groups and amino and/or hydroxyl groups of type I collagen, following reticulation. Amide bonds within peptides give rise to different infrared signals associated with bonds vibrations, however, in our case, there was no meaningful evidence of change in the spectra with HA addition with respect to just collagen. The only difference observed is that all bands related to amides were decreased in intensity after reaction with hyaluronic acid. All these signals can be related to triple-helical conformation of collagen fibrils as reported elsewhere ²⁷³; therefore, we supposed that our process did not significantly affect collagen structure. FT-IR analyses confirmed also that no other chemical reagents were embedded into the cross-linked scaffolds, after synthesis protocol. Degradation thermal profiles, reported in **Figure 33a**, documented a similar trend between scaffolds, especially in the first phase, confirming hygroscopic properties. However, the second and last stretch related to the HyCo curve seemed to differ slightly with respect to control, probably in response to HA incorporation within the collagen matrix. DSC analysis suggested that the thermal stability of collagen was improved after the cross-link reaction, as confirmed by the increase of temperature associated with the endothermic peak (**Figure 33b**). From SEM images, it should be noted that the surface and internal porosity of scaffolds were also affected by cross-linking due to the formation of smaller void areas. However, micrographs always showed an open and interconnected void structure, consisting of pores with an average diameter not smaller than $50\text{ }\mu\text{m}$ (**Figure 34a-f**) that is considered the boundary size for ensuring proper distribution and cell viability

²⁷⁴. Therefore, scaffold architecture could be suitable for stem cells or chondrocyte colonization. Moreover, cross-linking process strongly increased the mechanical strength of this system as documented by a ten times increase in Young's modulus and tensile strength at break, compared to pure collagen (**Figure 35a,b**). This result is extremely relevant suggesting the potential scaffold application as a cartilage substrate.

Concerning absorption capability, collagen-based materials are easily wettable by aqueous solvents, including biological fluids, for the presence of many polar functional groups capable of binding water. Following hydration phase, these polymeric materials can swell and maintain a fraction of water within their structure over time. On the other hand, swelling properties of materials have been demonstrated to significantly affect cell behavior including adhesion and proliferation. After 24 hours a low change in weight of absorbed saline solution by both tested membranes was recorded, suggesting that their maximum hydration degree was reached in a short time. However, the hybrid scaffold has shown the highest absorbency value after 24 hours (**Figure 36a**). Generally, the ability of water uptake by spongy scaffolds mainly depends on two factors: hydrophilicity of material and stability of its porous structure following water-soaked. In more detail, hydrophilicity is influenced by polarity of each macromolecular constituent and their crosslinks degree ²⁷¹. The presence of HA in scaffolds should promote intermolecular bonds by creating ion complexes between its carboxylic groups and the amino groups of collagen, thus diminishing the swelling ability. Furthermore, HA could also form ester-type bonds between its carboxylic groups and/or hydroxyl groups of collagen, in the presence of cross-linkers, in this way providing covalent attachment of HA to collagen. From the analysis of swelling behavior, cross-linking caused a reduction of this parameter proposing a more stable network reached following this reaction (**Figure 36b**). Following hydration phase, collagen tended to lose its original shape over time, this behavior might reduce its efficacy during *in vivo* applications. In contrast, HyCo seemed to maintain its native structure, demonstrating a good shape memory during the whole incubation time (**Figure 36c**). Therefore, we hypothesized that cross-linked

scaffolds could better maintain their structural integrity in biological fluids following *in vivo* implantation.

Another issue associated with clinical failure of collagen-based scaffolds is their extreme susceptibility to enzymatic degradation by collagenases, which makes faster its catabolism. For this reason, we decided to perform an *in vitro* enzymatic assay. HyCo degraded more slowly compared to collagen (**Figure 37a**). This peculiar behavior can be ascribed to cross-linking reaction with hyaluronic acid. Therefore, an increase in the structural stability of collagen by crosslinking reaction has proved a successful way to prevent the catabolism by collagenase. Finally, despite cells can be seeded in collagen scaffolds, it is always necessary to estimate if the crosslinking reaction may induce on cytotoxicity. *In vitro* cytotoxicity by using hPBMCs suggested that hybrid scaffold still keeps good biological characteristics after crosslinking and ensured sufficient cells vitality.

5.6 Conclusion

In this study, a novel hybrid scaffold, based on collagen and HA, was successfully synthesized by a chemical crosslinking reaction performed in heterogeneous phase. The novel scaffold showed strongly improved mechanical properties and higher resistance to enzymatic degradation, compared to commercial collagen with lower cytotoxicity. Described synthesis procedure is versatile and can be largely applied to different collagen structures. Moreover, characteristics of the obtained scaffold could further investigated to better ascertain its potential applications in cartilage defect repair.

CHAPTER 6: Conclusion

Articular cartilage lesions are still an open challenge in orthopedics because effective clinical solutions are not available yet, even though many advances in regenerative medicine and tissue engineering have been made, including autologous chondrocyte implantation using collagen matrices as adjuvants; however, an effective and durable chondro-integration is lacking. Moreover, chondrocytes isolation from cartilage biopsy and *in vitro* expansion are extremely delicate processes with high rates of failure, and hyaline cartilage biomechanical properties are still not reproducible *in vitro*. For these reasons, the development of novel advanced cellular models to investigate cartilage regeneration processes is becoming increasingly important. Human mesenchymal stem cells harvested from bone marrow or other sources could represent a valuable alternative to cartilage-derived chondrocytes; however, several technical issues are present, especially for promoting and maintaining chondrogenic phenotype over time, as well as to prevent hypertrophic shift. Indeed, bone marrow-derived mesenchymal stem cells do not show a successful chondrogenic differentiation using the conventional monolayer approach even using a chondrogenic culture medium supplemented with 10 ng/mL of human transforming growth factor (hTGF)- β 1. Using this standard approach, cells tend to de-differentiate with downregulation of all chondrogenic markers thus making this protocol not suitable for chondrogenic commitment studies. Conversely, 3D high-density cultures (spheroids) supplemented with 10 ng/mL of TGF- β 1 was more effective in promoting a marked upregulation of chondrogenesis-related genes, such as *SOX9*, *COL2A1*, and *ACAN*. Using this system, Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) also showed an effective chondrogenic commitment making this stem cell source as a potential source for tissue engineering. Cytokine expression analysis is frequently not investigated in studies on chondrogenesis; however, their secretion could be responsible for rapid differentiation through a paracrine effect. Indeed, we showed that IL-6 and IL-10 are upregulated during 3D cultures of both BM- and WJ-derived mesenchymal stem cells, suggesting their involvement in chondrogenic differentiation. Another frontier in tissue engineering is growth factor microencapsulation for controlled release and degradation prevention,

and supercritical emulsion extraction (SEE) technology is suitable for hTGF- β 1 encapsulation in poly-lactic-co-glycolic acid (PLGA), providing advanced control of carriers' size and morphology, as well as ensuring a constant growth factor release over time. Moreover, SEE-fabricated carriers do not have intrinsic toxicity and could be safely introduced in biomedical devices. Indeed, when hBM-MSCs were cultured in collagen hydrogels and in the presence of hTGF- β 1-loaded carriers under static or dynamic (perfusion) conditions, cells showed an effective chondrogenic commitment. This system could be used for simultaneous release of multiple growth factors and/or immunomodulatory signals to further explore new solutions for *in vitro* cartilage repair and regeneration. Indeed, collagen hydrogels enriched with PLGA microcarriers have a relatively low dynamic viscosity (104 Pas) and body temperature (37 °C) cross-linkage making this biomaterial a potential intra-articular injectable drug for controlled growth factor or small molecule delivery for *in vivo* cell differentiation. In addition, a novel hybrid scaffold based on collagen and hyaluronic acid was synthesized by a chemical crosslinking reaction performed in the heterogeneous phase showing extremely performant mechanical properties, high resistance to enzymatic degradation, and excellent cell viability. In conclusion, our works produced advances in *in vitro* cartilage tissue engineering and potential novel controlled drug delivery systems for articular cartilage injury treatment.

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