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**UNIVERSITÀ
DEGLI STUDI
DI TORINO**

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3D Collagen based biomimetic scaffolds

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Turin, March 16, 2021

Summary

Bone-tissue regeneration induced by biomimetic bioactive materials is the most promising approach alternative to the clinical ones used to treat bone loss caused by trauma or diseases such as osteoporosis. The goal is to design nanostructured bioactive constructs able to reproduce the physiological environment of the native tissue, therefore guiding cell behaviour during the regeneration process. In this scenario, composite and bioactive systems based on type I collagen represent promising tools to mimic the physico-chemical and nano-structural features of bone extracellular matrix, while 3D-printing and electrospinning technologies can support the design of complex structures limiting constraints of final shape and porosity. However, these fabrication techniques require a complex optimization of biomaterial formulations in terms of specific rheological and mechanical properties while preserving high biocompatibility.

In this context, the research activities conducted during this PhD, were mainly focused on the design of collagen-based scaffolds for bone tissue engineering applications, starting from the optimisation and characterisation of the material formulations up to the definition of the manufacturing process and the most suitable crosslinking strategy, finally exploring the bioactivity and biological response of the developed systems.

Initially, type I collagen, strontium containing mesoporous bioactive glasses (Sr-MBGs) and nano-hydroxyapatite, were combined to obtain homogeneous hybrid formulations, exploiting the advantages of the different materials in a unique system. Sr-MBG containing formulations were studied for the ability to combine the well-known high bioactive character of the inorganic phase with the release of pro-osteogenic strontium ions. In parallel, the homogeneous dispersion of nano-HA particles was explored by optimising the concentration of a cytocompatible dispersing agent. Moreover, a comprehensive rheological assessment was exploited to investigate the suitability of all the developed formulations for 3D extrusion printing applications.

Subsequently, different chemical crosslinking methods were investigated to define the most effective in inducing the formation of covalent bonds between

collagen molecules, aimed at increasing the insufficient mechanical properties and stability of the systems.

After the optimisation of the different collagen-based formulations, a few strategies were explored to design high resolution scaffolds by means of extrusion printing. In this frame, the freeform reversible embedding of suspended hydrogels (FRESH) method was exploited and optimised to enable the printing of the developed biomaterial inks.

An alternative method concerning the UV curing of the collagen-based systems was assessed to promote the preliminary stabilisation of the printed constructs while printing. To this aim, methacrylated type I collagen was used in combination with Sr-MBGs and nano-HA to obtain photocurable systems, where the concentration of the cytocompatible photoinitiator (Irgacure 2959) was optimised by means of a comprehensive rheological assessment.

In parallel with the development of materials and manufacturing processes, the cytocompatibility of the composites and the crosslinking treatments was assessed using pre-osteoblastic MG63 and Saos-2 cell lines. In a second phase, the optimised collagen-based systems and scaffolds were studied in presence of human osteoblast and osteoclast co-cultures, to explore their potential to promote cell viability, proliferation and differentiation.

Finally, the use of type I collagen was explored for the design of biomimetic scaffolds by means of electrospinning technologies. Accordingly, different solvents, collagen concentrations and process parameters were studied with a particular focus on the preservation of the protein supramolecular structure.

Subsequently, Sr-MBGs were combined with the optimised collagen solution to obtain composite nano-fibrous scaffolds.

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*For those who love
challenges*

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Thesis goals

Nowadays, bone defects and fractures represent one of the most relevant clinical problems, with data reporting more than two million bone grafts per year [1–3]. Considering the significant drawbacks and limitations shown by the gold-standard treatments, and the ever-increasing demand for bone substitutes, nowadays tissue-engineering therapies are considered a promising alternative strategy for bone reconstitution [4–8]. Especially in diseases such as osteoporosis, the design of biomaterials able to direct and promote bone tissue remodelling/regeneration rather than simply filling the bone defect and supporting the damaged tissue, is considered a crucial aspect [2,4]. In this frame, regeneration strategies based on biomimetic and bioactive materials that actively interact with the surrounding living tissues holds great potential for a more effective clinical treatment, at variance with the conventional passive biomaterials which often prevent any potential crosstalk with the body tissues [4,9,10].

The proper selection of biologically inspired biomaterials and fabrication processes is thus considered a key factor to design engineered constructs mimicking the complex nano-, micro- and macro-structures, as well as the natural mechanical and biological properties of the native tissue [4,11,12]. In the field of bone-tissue engineering (BTE), nanomaterials are exploited to design biomimetic constructs, considering that bone itself is characterized by a perfectly arranged nanocomposite nature [7,13]. However, the reproduction of the complex structure of bone at multiple scale levels, as well as the mimicking of its composition and biochemical cues, still represents a challenging target, and the ideal combination of materials and techniques has not yet been found [4,14]. Based on this, effective bone scaffolds should be designed as osteoconductive and osteoinductive constructs presenting both micro- and nano-architectures, to increase the structural biomimicry while enhancing the ability to stimulate bone regeneration [2,7,14].

Among the bioactive and biomimetic biopolymers, type I collagen, the main organic component of the bone extracellular matrix, is widely known to have distinctive biological properties, due to the high density of amino acid sequences that positively influence cell bio-recognition and adhesion [14,15]. However, the low biomechanical stiffness and the rapid degradation rates shown by *in vitro* reconstituted collagen matrices, highly limit the use of collagen in bone tissue engineering applications [16–18]. To overcome these issues, different studies reported improved stability and mechanical stiffness upon the combination of collagen with inorganic phases acting as reinforcing agents, as well as the use of different strategies for the chemical crosslinking [14,19,20].

Considering the natural composition of bone, hydroxyapatite particles or bioactive glasses are often exploited to create biomimetic composites as potential inorganic constituents [16,21]. Mesoporous bioactive glasses (MBGs) have largely been recognized as effective osteopductive materials thanks to their ability to

promote the formation of hydroxyapatite crystals, as well as the release of calcium and silicon ions as dissolution products, and to their very high specific surface area, which make them more reactive than traditional ones [22,23]. More recently, to empower the osteogenic potential of bioactive glasses, several studies have reported a beneficial effect of strontium ions on cell behaviour, mainly due to its similarity with calcium, by enhancing new bone formation while reducing osteoclast resorptive activity [24,25].

The combination with inorganic phases is known to provide enhanced mechanical stiffness and stability to collagen-based materials, but, by itself, it may be insufficient. Therefore, exogenous crosslinking methods are normally required to ensure the formation of a stable and stiff matrix composed by a dense fibrillar packing [14,20].

Besides the physico-chemical features, biomimetic constructs resembling the architectural feature of native bone at multiple length scales, represent a promising tool for guiding the proper tissue regeneration [4,14]. At present, additive manufacturing technologies are the only techniques able to accurately reproduce complex architectures without significant constraints over the final shape and porosity [26,27]. In addition, electrospinning techniques have been largely used to process polymeric based materials, thanks to their ability to design scaffolds mimicking the submicrometric fibrous structure of natural extracellular matrix [28–30]. However, the use of natural derived soft polymers such as collagen, and high concentrations of inorganic phases, still present several issues when using additive manufacturing and electrospinning approaches. In addition, according to the fabrication technique selected, the final features of the material need to be properly optimised and characterised, to achieve high resolution of the 3D structures, while preserving the physico-chemical and structural properties of the protein.

In this scenario, the research studies conducted in this PhD, aimed at exploring the use of type I collagen for the design of biomimetic scaffolds able to reproduce at best the native features of bone tissue, mimicking its composite nature and its complex hierarchical architecture by optimising innovative manufacturing strategies.

Most of the research activities were conducted in the frame of the ERC BOOST Project (Biomimetic trick to re-balance Osteoblast-Osteoclast loop in osteoporosis treatment: a Topological and materials driven approach; ERC Consolidator Grant 2015, GA 681798), aimed at designing biomimetic bone-like scaffolds able to provide biochemical and structural stimuli to rebalance osteoblast (OB) and osteoclast (OC) activities in the case of osteoporosis.

As first goal, different collagen-based formulations combining type I collagen, strontium containing mesoporous bioactive glasses (Sr-MBGs) and rod-like nanohydroxyapatite (nano-HA) were optimised to obtain homogeneous hybrid formulations, as described in Chapter 3. The uniform distribution of the inorganic phase particles was investigated alongside the reconstitution of the collagen fibrils upon physiological pH and temperature (pH 7.4; 37 °C). In particular, the proper

dispersion of nano-HA was induced by optimising the concentration of a cytocompatible dispersing agent, i.e., Darvan 821-A. Moreover, the preservation of the bioactive properties of Sr-MBGs was investigated after addition into the collagenous matrix, observing the deposition of HA crystals upon incubation in Simulated Body Fluid (SBF).

A comprehensive rheological study was performed to explore the potential use of the developed collagen-based formulations as biomaterial inks for extrusion printing. In details, the variation of suspension viscosity at 10 °C was studied alongside the sol-gel transition and the visco-elastic properties of the systems at 37 °C.

The second goal of the study concerned the improvement of the visco-elastic properties and stability of the collagen-based systems, exploring different exogenous crosslinking methods. Accordingly, in the second part of Chapter 3 are discussed the assessment of different crosslinking methods and the further selection of the most efficient strategy, based on the physico-chemical and rheological properties of the composite materials alongside the cytocompatibility evaluation (reported in Chapter 5).

In details, among the most cytocompatible chemical crosslinking agents of collagen reported in the literature, the influence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) and N-hydroxysuccinimide (NHS), 4-star poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG) and genipin (GEN), was investigated by observing the resulting material strength, degradation rate and cytocompatibility, compared to the non-crosslinked materials. Moreover, the potential release of strontium during the chemical treatments was investigated in the case of Sr-MBG containing systems, where alternative crosslinking media were evaluated in order to hinder the premature massive loss of the pro-osteogenic ions.

After the optimisation of the material formulations and the identification of the most suitable crosslinking chemical treatment, the printability of the developed hybrid systems was explored by using a commercial 3D Bioprinter (BIOX, CELLINK). Despite the opportunity to obtain scaffolds with higher control over the final porosity and architecture compared to conventional manufacturing techniques, the extrusion printing of highly complex nanostructures requires the use of biomaterials characterized by specific properties, such as shear thinning to enable material extrusion through very thin needles as well as yield stress and fast shear recovery after deposition to avoid structure collapse [31]. According to the rheological properties of the collagenous biomaterial inks, different printing strategies were explored and optimised to enable the extrusion printing of the soft materials by using 27 G and 30 G needles, with the aim to obtain high resolution and shape fidelity of the resulting scaffolds, as described in Chapter 4.

Based on the recent study reported by Hinton and co-workers [32], the extrusion of the developed hybrid formulations into gelatine-based baths was investigated, according to the freeform reversible embedding of suspended

hydrogels (FRESH) method. After printing, the incubation of the scaffolds at 37 °C was exploited to induce the gelification of the printed constructs while removing the supporting material. Different gelatine slurries were explored and compared for their ability to promote high shape fidelity while supporting the material deposition. Moreover, the previously optimised crosslinking treatment was used to improve the final mechanical strength and stability of the resulting scaffolds, observing the potential influence over the final morphological features.

As alternative printing strategy, UV curable methacrylated collagen-based hybrid formulations were developed to promote the preliminary crosslinking of the scaffolds while printing. Rheological tests were performed to study the crosslinking kinetics and the visco-elastic properties of the photo-curable systems, while optimising the concentration of the cytocompatible photoinitiator Irgacure 2959. In parallel, STL files derived from nano-computed tomography of human bone samples and reproducing its natural trabecular architecture, were developed to promote the simultaneous deposition of biomaterial ink and supporting material to enable the UV curing of the scaffold layer-by-layer.

As described in Chapter 5, thanks to the collaboration with Istituto Ortopedico Rizzoli of Bologna (Scienze e Tecnologie Biomediche, IRCCS) and Università Politecnica delle Marche (Department of Clinical and Molecular Sciences – DISCLIMO), the cytocompatibility of the developed formulations and crosslinking methods was assessed by using pre-osteoblastic MG-63 and Saos-2 cell lines.

Moreover, thanks to the protocols optimised in collaboration with Istituto Ortopedico Rizzoli and under the supervision of Dr. Gabriela Ciapetti, indirect co-culture systems of human osteoblasts (OBs) and osteoclasts (OCs) were exploited to preliminary study the influence of the different bioactive composites and scaffolds over cell adhesion, proliferation, and differentiation.

Finally, according to the main goals of the GIOTTO European Project (Active aGeIng and Osteoporosis: The next challenge for smarT nanobiOMaterials and 3D technologies; NMBP-22-2018, GA 814410), hybrid formulations based on type I collagen and strontium-containing MBGs have been optimised and characterised with the aim to create flexible nanostructured matrices by means of electrospinning technologies, and the results are reported in Chapter 6. The experiments were conducted in collaboration with the Institute for Technology-Inspired Regenerative Medicine (MERLN) under the supervision of Prof. Lorenzo Moroni, during the months spent at the Maastricht University.

In view of the different studies in the literature reporting the denaturation of collagen when processed by means of electrospinning techniques [33], the use of bovine type I collagen for electrospinning of bone scaffolds was explored by testing different solvents and protein concentration. The preservation of the physico-chemical and structural properties of the protein, alongside the morphological features of the resulting scaffolds were investigated. These preliminary experiments were exploited to demonstrate the opportunity to design composite fibrous scaffolds containing bioactive inorganic phases such as Sr-MBGs.

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CHAPTER 1

Biomimetic approaches for bone tissue regeneration

1.1 Introduction

The present research study aimed at exploring the use of type I collagen for the design of 3D biomimetic scaffolds for bone tissue engineering applications by means of additive manufacturing and electrospinning technologies. Different collagen-based hybrid formulations have been developed combining type I collagen derived from bovine Achilles tendon with strontium containing mesoporous bioactive glasses (Sr-MBGs) and nano-hydroxyapatite (nano-HA). The developed materials were fully characterised to investigate their suitability for extrusion 3D printing applications and electrospinning technologies. Different strategies were explored to produce biomimetic composite scaffolds reproducing the morphological features of bone extracellular matrix at the micro- and nanoscale. Cytocompatible crosslinking methods were optimised to increase the material strength and stability, favouring the formation of covalent bonds between collagen molecules. Finally, a preliminary biological assessment was performed on the developed materials and scaffolds, in presence of osteoblast precursor cells and co-culture of human osteoblast (OBs) and osteoclasts (OCs), with the aim to investigate their potential use for bone tissue regeneration applications.

According to the main goals of the research studies and with the aim to provide a general overview of the state of the art, this introductory chapter will present the gold standard strategies and the recent advances in bone tissue regeneration, with a special focus on the use of type I collagen to design biomimetic materials and scaffolds. In detail, Section 1.2 will be dedicated to bone tissue engineering, introducing the main features of bone and the mechanisms behind its continuous regeneration, as well as the potential alterations due to pathological conditions such as osteoporosis. Moreover, the current approaches exploited to promote bone regeneration will be discussed, describing advantages and limitations of bone scaffolds and their potential combination with co-culture systems to produce biomimetic models.

The key role of type I collagen in bone tissue engineering will be reported in Section 1.3, presenting the main characteristics of this structural protein, its use either alone or in combination with additional bioactive phases such as MBGs and

nano-HA and the current strategies used to increase the final stability of the collagen-based constructs.

Finally, the fabrication of bone scaffolds will be presented in Section 1.4, demonstrating the superior potential of additive manufacturing technologies and electrospinning in designing biomimetic scaffolds, also discussing the main advantages, limitations, and current achievements in bone tissue regeneration applications.

1.2 Bone tissue engineering (BTE)

Bone disorders and injuries represent a significant healthy and economic burden affecting millions of people worldwide, that is likely to worsen because of the increasing aging of population and the diffused sedentary lifestyle. Where pharmacological treatments are not sufficient, the partial or total replacement of the compromised tissue is required, leading to a significant reduction of life quality alongside considerable socioeconomic impact. Despite surgical practises have demonstrated remarkable progress leading to the improvement of clinical outcomes, these approaches are limited by the poor availability of autologous tissues and the significant donor site morbidity [1].

Consequently, in the last three decades, bone tissue engineering has gained great interest as alternative method for the regeneration of healthy bone tissue, promoting the restoration of its function by means of biocompatible and bioactive constructs able to stimulate specific biological responses while being integrated and replaced by regenerating tissue [2,3].

Biocompatible and biodegradable scaffolds seeded with bone cells *in vitro* can be implanted immediately or after predefined culture periods to promote the regeneration of new tissue, exploiting the synergistic effect of biomaterials, cells, and bioactive cues[4].

In this frame, the chemistry as well as the architectural features of the scaffolds, both at the micro- and nanoscale, are considered fundamental factors to properly guide the cell response in the engineered 3D environment [4,5].

One of the first goals in tissue engineering is therefore the faithful reproduction of the tissue micro-architecture mainly represented by its extracellular matrix (ECM), that dictates the mechanical and biological behaviour and regulate cellular activity by direct or indirect actions. Since this biological environment is unique for each tissue, scaffolds for bone tissue engineering should mimic at best the composite features as well as the multi-scale hierarchical organization of bone [6–8].

1.2.1 Physiology of bone tissue

Bone is a highly complex and hierarchically organised structure that can be classified as micro- and nano-composite tissue. The extracellular matrix (ECM) is mainly represented by type I collagen molecules produced by osteoblastic cells and assembled in fibrous matrix able to provide the native scaffolding material for bone

formation. Osteoblasts (OBs) are also responsible for the subsequent secretion of amorphous calcium phosphates into the collagenous ECM, subsequently organised in nanosized particles that accommodate the gaps between collagen molecules, finally forming hydroxyapatite nanocrystals (nano-HA). The nano-HA particles arrange and crystallise not only within but also between collagen fibrils, in a process named as intrafibrillar and interfibrillar mineralisation [2,9,10].

Type I collagen counts for about 30-40% of the dry weight, and together with water and few other non-collagenous proteins, provide a viscoelastic and tough matrix, structurally reinforced by the mineral phase represented by calcium phosphates (about 60-70 wt%). As a matter of fact, the bone matrix represents an important mineral deposit, affecting and regulating the calcium and phosphate homeostasis in the circulating body fluids [1,11].

Type I collagen and nanohydroxyapatite crystals are assembled and organised in lamellae and haversian systems to build the basic structural units of bone ECM, the osteons, as shown in Figure 1.1.

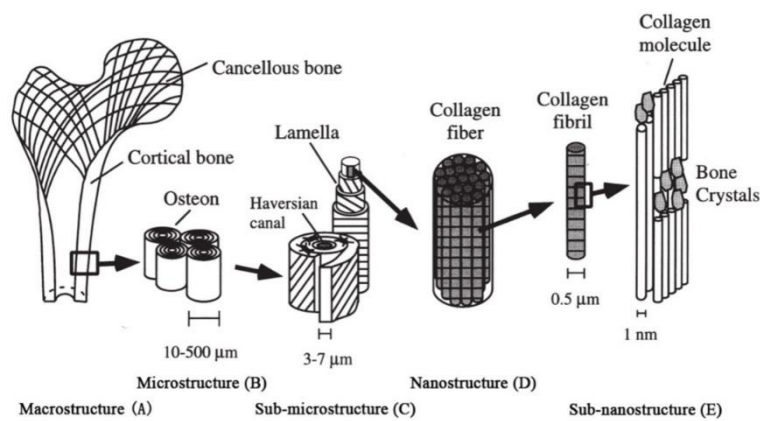


Figure 1. 1 Multi-scale hierarchical organization of bone [1,10].

The osteon thus consists in several lamellae wrapped in concentric layers forming a central haversian canal, that contains nerve and blood vessels. The macrostructure of bone is consequently obtained by the assembly of osteons, obtaining trabecular struts and dense cortical bone [1,4,5].

The composite nature and the structural organisation dictate the peculiar mechanical properties of bone, characterised by high compressive, flexural, and tensile strength, that significantly change between cortical and cancellous tissue, as summarised in Table 1.1.

This complex architecture is constantly renewed thanks to the balanced activities of osteoblasts (OBs) and osteoclasts (OCs), that together with osteocytes and bone lining cells, represent the cell population of bone. The constant deposition by OBs and resorption activity by OCs determine the dynamic character of bone tissue, subjected to a constant state of renewal in the well-orchestrated process of bone remodelling, where fracture or defect healing are significantly influenced by the applied mechanical stimuli [1,4,12].

Table 1. 1 Mechanical properties of cortical and cancellous bone.

Property	Cortical bone	Cancellous bone
Compressive strength (MPa)	10-230	2-12
Flexural, tensile strength (MPa)	50-150	10-20
Strain to failure (%)	1-3	5-7
Young's Modulus (GPa)	7-30	0.05-0.5

Bone remodelling

Bone remodeling is a continuous regenerating process performed by specialized cells which are bone resorbing osteoclasts (OCs) and bone synthesizing osteoblasts (OBs), assembled in the basic multicellular unit (BMU) together with osteocytes and bone-lining cells. Multiple bone remodeling events occur at the same time at different sites of the body, leading to a complete functional renewal of the skeleton every ten years [13,14].

In physiological conditions, the remodelling mechanism, schematically represented in Figure 1.2, starts with the bone resorption phase, regulated by OCs [15]. In particular, OC precursors are activated by an increased production of receptor activator of nuclear factor- κ B (RANK) ligand (RANKL) by osteocytes and osteoblasts in response to tissue microdamage or endocrine stimuli, such as an increased parathyroid hormone concentration associated with hypocalcaemia. The newly formed RANKL binds to its receptors on OC precursor cells, stimulating their fusion in a syncytium and, consequently, the formation of a multinucleated mature OC [16].

To promote the adhesion of these cells on the bone surface and the consequent implementation of their resorption action, the lining cells present at the bone surface form a raised canopy over the site to be resorbed [17].

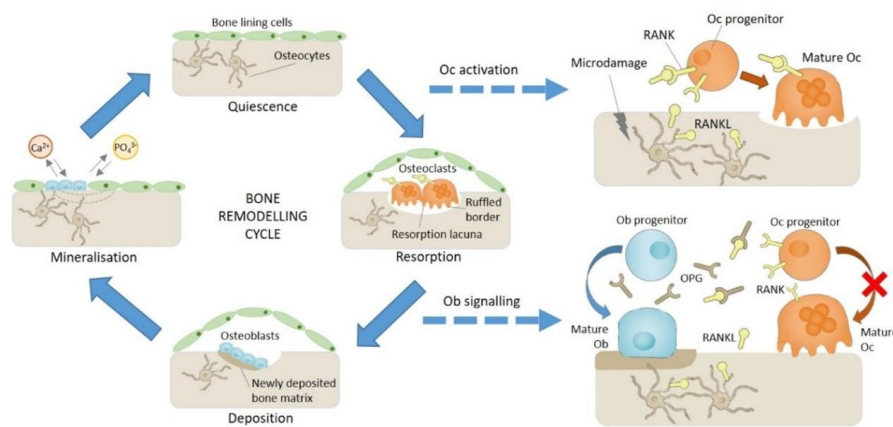


Figure 1. 2 Schematic representation of the bone remodelling process [15].

After adhesion, activated OCs start to resorb mineralized bone and organic matrix for 2-4 weeks before undergoing apoptosis. OCs can dissolve the bone mineral phase by creating an acidic environment in the sealed zone, characterised by the coupled introduction of H^+ ions, by means of the H^+ -ATPase pump and Cl^- ions, through their chloride channels, with the consequent formation of hydrochloric acid. Subsequently, the collagen-based matrix is degraded by metalloproteinases and proteases, such as cathepsin K, creating a lacuna on the bone surface. At the end of the resorption phase, OCs undergo a programmed death and the tissue present at the surface of the newly created lacuna is prepared to allow OB adhesion and the following bone formation phase [17].

The intermediate period between OCs resorption and the start of matrix deposition by OBs is called the reversal phase, where the signals derived from matrix resorption induce new bone formation.

The last stage is the formation phase, where OBs synthesise and deposit the osteoid, a matrix rich in type I collagen that will be successively mineralised through a series of phosphate and calcium ion exchanges with the extracellular environment [18]. Eventually, a limited number of OBs surrounded by mineral matrix undergo terminal differentiation and become osteocytes. In approximately 4-6 months the last phase of the process is concluded and about 50-70% of OBs undergo apoptosis while the others become bone-lining cells or osteocytes [17,19–21].

Despite OCs and OBs are not placed in direct contact in the BMU, their activities are coordinated, as proved by the physical connection existing between resorption and new bone formation through the reversal phase [11,22].

This dynamic and complex process of bone remodeling is strictly regulated by different mechanisms acting as local and systemic regulation [23,24].

During the fracture healing or remodeling process, as well as bone formation during the skeletal development, another important role is covered by vascularization and oxygen contribution. Bone is a highly vascularized tissue with a large number of vessels and capillaries directly participating to the osteogenic generation of new bone, as demonstrated in bone fractures, where the growth of

blood vessels and the recruitment of osteoprogenitors are coupled. Endothelial cells and osteolineage cells are often juxtaposed, with skeletal cells secreting angiogenic factors, whereas endothelial cells (ECs) produce angiocrine factors such as Vascular Endothelial Factor (VEGF) and chemokines that regulate skeletal cell behavior [25]. The evidence that alterations of the complex biochemical interactions between vasculature and bone cells may lead to various clinical manifestations further proves the essential role of vascularization in osteoinduction [26].

In addition to vascular network, other external factors that take part in the complex coordination of bone cells are the local micro-mechanics. Bone cells are constantly exposed to mechanical stimuli and the bone mass is preserved thanks to the mechanical loading. The mechanical stimuli are primarily captured by osteocytes and further transmitted, mainly through OBs and bone-specific mesenchymal stromal cells (MSCs) which cooperate in inducing bone formation [27].

Generally, in adults, the relatively low turnover rate of cortical bone is about 2%-3% per year, enough for maintaining adequate biomechanical properties. On the contrary, the rate for trabecular bone is higher and counted to be about 25%, underlining the importance of its contribute to the mineral balance [21,28].

In this scenario, any potential alteration of the reported regulatory mechanisms and communication pathways can lead to the imbalance between cell activities, finally compromising the overall quality of regenerating bone tissue.

Alterations of bone remodelling in osteoporosis

The overall result of the altered remodelling process is mainly the modification of the bone structure that involves both the trabecular and the cortical tissues, consistent with the progression of pathological conditions such as osteoporosis (OP). It is possible to affirm that OP is responsible for an overall reduction of bone quality, leading to decreased bone strength and significant architectural and compositional alterations in both the trabecular and cortical compartments when compared to healthy tissue [15,29].

Nowadays, osteoporosis represents a worldwide skeletal disease affecting the elderly population, with a very high incidence in post-menopausal women. The reduction of bone mass due to an imbalance in the remodelling process toward the resorption phase is associated with an increased risk of bone fractures with a consequent decline of patients' quality of life [30,31].

Considering the numbers, worldwide, 8.9 million osteoporotic fractures occur annually, more than 1000 fractures every hour, with a remarkable cost for society and health care systems. Moreover, due to the progressive population aging, the incidence of osteoporotic fractures in the European Union (EU) is expected to double in few decades.

Although the higher incidence in women, OP has been demonstrated to affect one out of five men above fifty years old, often presenting even significantly worse

prognosis. Recent data also showed that, in the EU, approximately 22 million women and 5.5 million men aged between 50–84 years may be affected by OP in the near future [32]. Since the total population's age along with the life expectancy of the elderly are estimated to increase by more than two-fold over the next 25 years, osteoporotic fractures still represent an important clinical issue with a significant impact on the cost for health care systems [15,32].

During the early stage of OP, the trabecular bone, characterised by a high surface area, is the one most affected by the surface-dependant resorption process. The osteoclastic resorption initially leads to the perforation of the trabeculae and successively to their complete removal, as schematised in Figure 1.3. With the further progression of the disease, the bone loss also involves the cortical bone, causing a reduction in the cortical thickness and an increase in its porosity [15,33,34].

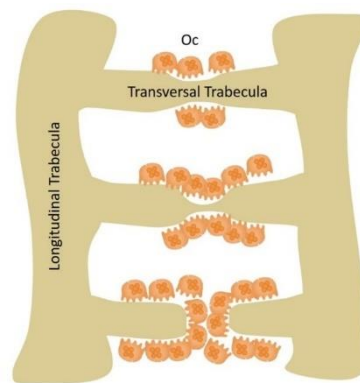


Figure 1. 3 Schematic illustration of the resorption and subsequent perforation of a trabecula by osteoclasts [15].

In physiological conditions, the bone resorption rate equals the formation one, but, in the presence of OP, these two processes become unbalanced toward bone resorption, causing bone loss. The imbalance between OB and OC activities is the result of altered cell signalling mechanisms that derive from several factors, such as a reduced production of oestrogens in post-menopausal osteoporosis, the increased secretion of parathyroid hormone (PTH) and reactive oxygen species concentrations in age-related osteoporosis, along with excessive thinness, alcohol and corticosteroid use [17,34]. Normally, oestrogens limit the production of RANKL and induce the secretion of osteoprotegerin (OPG) from OBs, a soluble decoy receptor that binds to RANKL in an antagonistic way to the RANK receptor, therefore modulating the OC signalling downstream of RANK. On the other hand, an oestrogen deficiency is associated with an increased osteoclast differentiation and resorption activity due to the higher secretion of RANKL from OB and with impaired mechanosensing by osteocytes [33]. Oestrogen deficiency is the main condition leading to the onset and fast progression of osteoporosis in the trabecular compartment of women in their first 3–5 years of menopause. For this reason, the oestrogen-dependent bone loss is defined as postmenopausal osteoporosis and it can lead to a high number of fractures, especially in bones characterised by a higher trabecular fraction (e.g., vertebral bodies) [33]. The lower incidence of fractures in

age-matched men is obviously explained by the absence of this marked oestrogen reduction in men. Moreover, different studies reported a more marked reduction of bone volume fraction in different osteoporotic sites especially in female patients [15].

However, after this rapid phase, a slower but longer age-related bone loss occurs, affecting both women and men and involving both the trabecular and the cortical bone compartments. This age-related osteoporosis is mainly correlated to vitamin D deficiency and a consequent increased level of parathyroid hormone (PTH) [35]. In fact, PTH promotes Oc activity and, consequently, bone resorption through the stimulation of RANKL expression and the reduction of OPG release by OBs [36].

1.2.2 Current treatments for bone regeneration

In the case of significant loss or damage of bone tissue following trauma or pathological conditions, spontaneous bone healing may be compromised resulting in patient morbidity and significant health care costs [37,38].

Conventional bone grafting procedures are the preferred surgical treatments, with bone autografts representing the gold standard for bone augmentation, and xenografts, allografts or synthetic bone substitutes as suitable alternatives in reconstructive orthopaedic surgery [39]. The treatment with autologous tissue is considered the “gold standard” procedure, since bone represents a natural osteoconductive and osteoinductive scaffold able to promote the ingrowth, proliferation and differentiation of cells necessary for bone regeneration [1,31]. To reduce the harvesting of autologous bone graft, the “Masquelet technique” and the Reamer-Irrigator-Aspirator (RIA®)-System have been reported among the most effective clinical practices. The first method consists in the preliminary use of a cement spacer and external fixators to avoid the collapse of soft tissues in the surgical cavity, filled after 6-8 weeks with morcellised cancellous bone graft. On the contrary, the RIA method provides an harvesting source alternative to the iliac crest, exploiting irrigation and aspiration during intramedullary reaming and collecting bone, that proved to be rich in stem cells, osteogenic cells and growth factors [1].

Despite the quite satisfactory clinical results of the current bone regeneration/augmentation methods, several limitations mainly arise from the poor availability of autologous tissue and the additional surgical procedures required for the harvesting that cause donor site morbidity [1,4,5].

Considering the significant drawbacks and limitations shown by the gold-standard treatments, and the ever-increasing demand for bone substitutes, nowadays tissue-engineering therapies are considered a promising alternative strategy for bone reconstitution [40–43]. Especially in diseases such as osteoporosis, the design of biomaterials and constructs able to direct and promote bone tissue remodeling/regeneration rather than simply filling the bone defect and supporting the damaged tissue is considered a crucial aspect [31,40,42,44].

The bone tissue engineering approach relies in the use of 3D scaffolds in combinations with cells and bioactive molecules to create an “osteogenic” substitute for bone diseases or defects [45–47]. A number of scaffolds mimicking the structural, mechanical, and biological properties of natural tissues have been developed in order to support new tissue formation when combined with cells [48,49]. Indeed, the development of an artificial ECM to be colonized by autologous or allogenic cells may allow to recreate a “patient-specific biological substitute” as close as possible to natural bone tissue [50].

In this scenario, the setting up of co-cultures of OBs and OCs have demonstrated to provide an *ex vivo* system for an in-depth examination of the cross-talk between bone cells and of their signaling pathways during the remodeling process [11]. To date, OB-OC co-culture systems have been adopted for several targets, such as the set up of the experimental model *in vitro*, a better understanding of the bone remodeling process, the efficacy of bone drugs or treatments and the development of regenerative strategies. As a consequence, different experimental protocols have been developed and explored with the aim to foster bone engineering techniques through a better understanding of the coupling mechanism acting in bone remodeling [11,51].

Despite the positive implementation of the co-culture system with functional materials providing a more reliable 3D environment, the faithful reproduction of the complex microstructure of the actual ECM still represents a significant challenge, whereas the use of additive manufacturing technologies as well as decellularized ECM have been proposed as advanced and promising tools to design well-organized scaffolds with a high degree of complexity and precision [162].

Scaffolds for bone tissue engineering

The use of multifunctional scaffolds for bone grafting and regeneration is increasingly gaining interest as advanced regenerative devices. Bone scaffolds are conceived as 3D supporting structures intended to restore both morphology and function of the natural bone tissue after severe trauma or pathological conditions resulting in skeletal non self-healing defects [38,53].

The design of bone scaffolds can be envisioned for *ex vivo* tissue engineering techniques or in alternative for *in situ* tissue regeneration. In case of *ex vivo* applications, the scaffold is used as 3D substrate to prefabricate the regenerating tissue that will be subsequently implanted into the targeted defect. On the other hand, the development of scaffolds for *in situ* bone regeneration has recently experienced significant advances due to the optimisation of rapid prototyping techniques that enable the design of *ad-hoc* structures with high control over the final morphology and pore architecture, also reducing the processing time [53–55].

In the last decades, several studies aimed at exploring the ideal characteristics of scaffolds for bone tissue regenerations, investigating different physico-chemical and structural properties to enhance cell proliferation and differentiation, as schematically represented in Figure 1.4 [4].

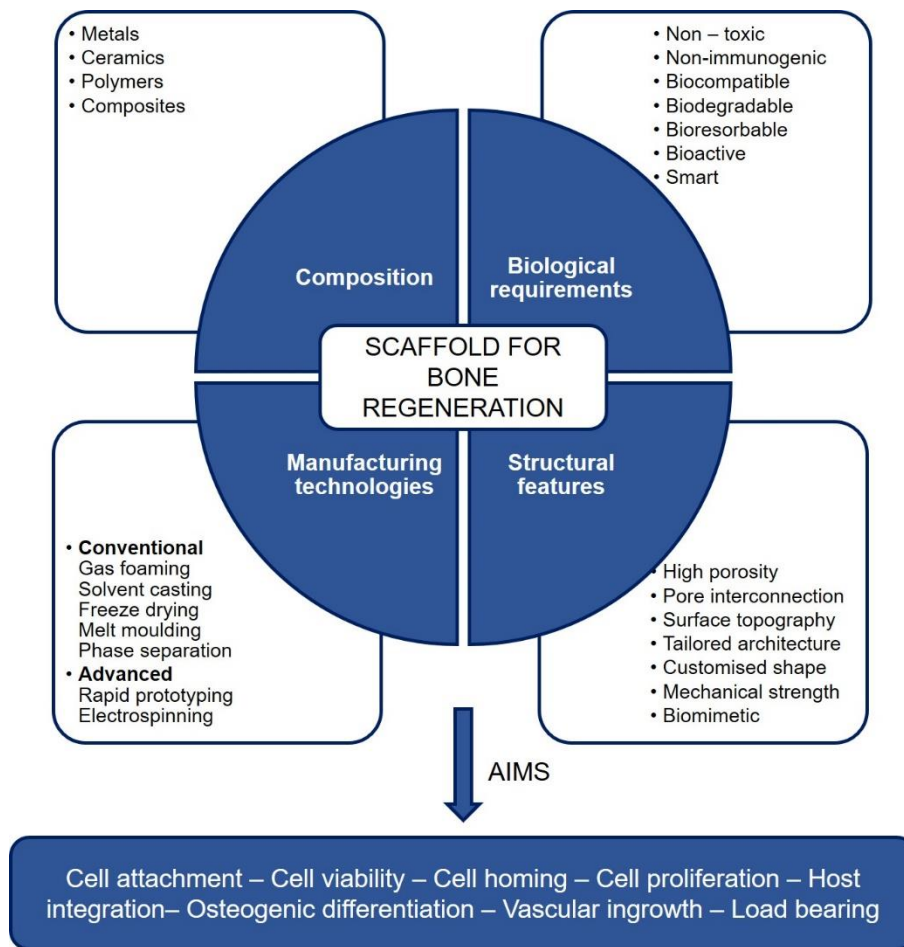


Figure 1. 4 Properties of scaffolds for none tissue regeneration.

In terms of architecture, the ideal bone scaffold should present an interconnected macro-porous structure, with a pore size ranging between 100 and 1000 μm , in order to promote the bone ingrowth, the diffusion of nutrients and metabolic waste products as well as the formation of a proper angiogenic network [1,56].

Beside the specific porosity, the overall architecture, mechanical properties, and composition of the scaffold play a key role in the success or failure of the implant. In this frame, the scaffold must guarantee the cellular adhesion, spreading, proliferation and function as well as direct the proper differentiation of primary cells. Furthermore, the degradation and release products should not only be fully biocompatible but also preferably act as cell differentiation stimuli towards osteoblast phenotype [6,31,57].

From the 1990s onwards, the introduction of additive manufacturing (AM) techniques in the medical field provided a potent tool to produce custom-made 3D models better resembling the architectural features of the native tissue. In contrast to conventional techniques such as melt moulding, freeze drying, solvent casting and particulate leaching, phase separation or gas foaming, AM technologies enable higher control over the final morphology and porosity of the scaffolds while

significantly reducing the use of organic and toxic solvents that hinder their biocompatibility [1,5,58].

Alongside the morphological features of the scaffolds, the constituent materials highly influence the interaction with the biological environment and dictate their final physico-chemical, mechanical and degradation properties. In this frame, the significant drawbacks presented by metal and ceramic implants in terms of cytocompatibility and biodegradability led to an increased interest towards composite systems combining the advantages of two or more materials while enabling a better control over the final mechanical and degradation features. Particularly, polymeric and ceramic composites have been widely suggested as promising systems, actually mimicking the nature of bone tissue. Polymeric scaffolds containing either calcium phosphates or bioactive glass particles, characterised by different shape and size, have proved to enhance mechanical integrity, bioactivity and expression of osteogenic factors [56,59,60]. Aliphatic polyesters such as poly(caprolactone) (PCL), poly(lactic-acid)(PLA), poly(glycolic-acid)(PGA) and their copolymers are among the most used synthetic polymers for the design of constructs for bone tissue regeneration. However, the degradation by-products and the lack of bioactive moieties able to promote cell adhesion, significantly limit their potential as regenerating materials [1,4,5].

On the contrary, the use of natural polymers in hybrid systems for bone tissue engineering enables the design of more biomimetic materials able to better reproduce the natural ECM of the regenerating tissue. Several natural polymers, often considered for the fabrication of bone scaffolds, such as gelatine, collagen, fibrin and silk fibroin can reconstitute a sub-micrometric fibrous matrix that better mimic the micro and nano-architecture of natural ECM [5,61]. Most of the natural polymers form hydrogels, well-known for their ability to retain great amounts of fluids and thus representing a proper environment for cell growing and proliferation, allowing the appropriate diffusion of oxygen and nutrients as well as cell migration. However, these natural derived matrices show poor mechanical properties and rapidly degrade if not properly cross-linked, resulting unsuitable for bone tissue repair [6]. According to these observations, their combination with synthetic polymers or inorganic phases is required for the design of stable 3D bone scaffolds, exploiting the bioactive features of biopolymers and the reinforcing effect of additional phases [61]. The proper integration of the inorganic phase into the polymeric matrix is also considered a key factor for the further mechanical stability of the constructs [5,62].

Moreover, the use of either therapeutic agents or growth factors such as transforming growth factor-beta (TGF- β), insulin-like growth factor (IGF), epidermal growth factor (EPG) and bone morphogenic protein (BMP), have been proposed to increase the osteoinductive character of the scaffold, as well as promoting angiogenesis [56].

To date, despite all the considerations and technological advances, the optimal scaffold for bone tissue regeneration still remains an unmet target, where different research studies are focused to design well-orchestrated multiphasic systems based on biocompatible materials and bioactive factors [4,5].

Co-cultures of osteoblasts and osteoclasts

The importance of the cross-talk between OBs and OCs and the advantages of studying both of them in a “all in one system” were already theorised by Rodan and Martin in 1981, who underlined the contribute of OBs in favouring the differentiation and activation of OCs through the release of factors able to bind to OC precursors, pointing out the need of OB cooperation to get functional OCs [63–66]. As confirmed by different research studies, the RANKL expression by OBs as signaling molecule involved in RANKL-RANK interaction for OC differentiation is the main regulatory mechanism of OB/OC coupling [67]. With the progression of the regenerative medicine strategies and applications to replace or repair the injured tissues, the cell co-culture system was confirmed to be a useful *in vitro* tool to unravel tissue mechanisms. Moreover, the precious contribution of OCs in bone remodeling was evidenced in different studies, whereas for long time BTE applications were primarily focused on the role of OBs in the bone formation process in presence of various scaffolds [68].

In this context, the use of scaffolds able to provide mechanical support and biological cues can guide the activity of bone cells, promoting the deposition of new ECM, overall mimicking the remodeling process *in vitro*. The combination of 3D constructs and cell co-cultures thus represents a key strategy to more closely resemble the *in vivo* regenerative situation [161]. According to Goubko et al., the self-assembly of multiple cell types into functional tissues should be encouraged by patterning two or more types of cells on scaffolds exploiting different manufacturing techniques with the final goal of improving control over the cell microenvironment *in vitro* through the spatial localization of cells on a designed substrate [163].

The early OB/OC co-culture studies frequently employed mouse-derived cells or cell lines due to the easy access and repeatability of results in experiments. More recently, several experimental OB/OC co-culture systems with human cells have been designed to simulate *in vitro* the skeletal remodeling process. Even if the use of human cells implies a large variance of donor sex, age and health, the outcomes generally confirmed the data from rat/mouse cell protocols [11].

Despite the large variability of protocols, materials and cell source reported in the literature, the successful use of co-culture systems as effective *in vitro* models for bone remodeling and bone tissue engineering applications have been already demonstrated [11,71–73]. As a matter of fact, Domaschke and co-workers reported the successful differentiation and subsequent mineralization of buffy coat-derived monocytes (Mc) and mouse ST-2 osteoblastic cells (OB) seeded onto mineralized collagen scaffolds in presence of osteogenic medium, where the degradation of the mineralized collagen matrix after 4 weeks by OCs and the subsequent formation of new ECM by OBs was documented [71].

The superiority of co-culture systems in mimicking and investigating the remodelling process was also demonstrated in the long-term study (8-32 weeks) conducted by Hayden R.S. et al. [74], where the co-culture of human mesenchymal

stem cell-derived OBs and THP-1-derived OCs onto silk films were compared with OB- or OC-single cultures. Results demonstrated as increased ECM deposition, as well as increased roughness parameters and mineral clustering throughout the 32 weeks of culture in case of OB/OC co-cultures compared to the mono-culture of OBs.

OB/OC co-cultures were also demonstrated to be a useful tool for the assessment of potential positive contribution provided by ECM-mimicking components or biomimetic modifications on designed constructs. Recently, Hammerl and co-workers studied the difference between medical grade polycaprolactone (PCL) scaffolds before and after the coating with a thin layer of calcium phosphate (CaP), comparing the single culture and the co-culture of OBs isolated from the femur head or shoulder with peripheral blood mononuclear cells (PBMCs) up to 63 days, as shown in Figure 1.5 [75]. PCL-CaP scaffolds proved to be superior to PCL alone in terms of OB and PBMC colonization, where OB proliferation and ECM deposition was favoured in the presence of PBMCs.

Overall, all the data reported in the literature highlight the relevance of OB/OC co-cultures as reliable tools to explore the biological mechanisms and cues behind the physiological cross-talks of OBs and OCs, as well as cost-effective *in vitro* platforms for the screening of scaffolds intended for bone regeneration applications [11].

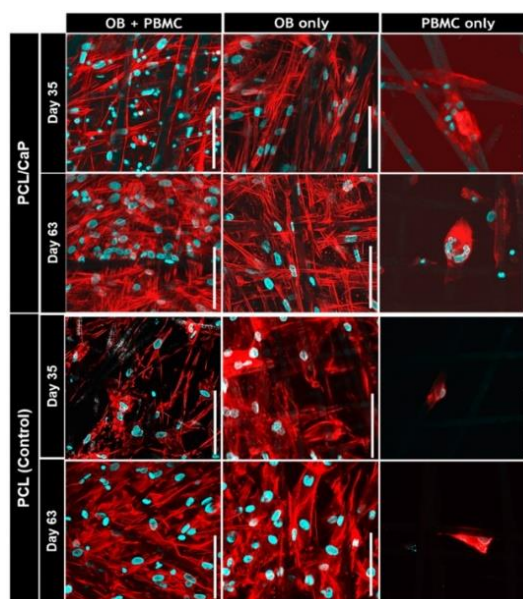


Figure 1. 5 Hoechst (blue-cell nuclei) and Phalloidin (red actin filaments) staining on cell-seeded PCL and PCL/CaP scaffolds in single culture and co-culture of OBs and PBMCs [75].

1.3 Type I Collagen as biomimetic material in BTE

The main goal of bone tissue engineering is to develop substitutes able to mimic the natural features of bone extracellular matrix to promote the regeneration of functional tissue. According to this, type I collagen, the most abundant component of the native ECM of different connective tissues, may be used in several applications as biomimetic scaffolding material, providing a biologically functional

platform presenting a unique multi-scale organisation [8]. The natural abundance of this protein, extracted from different animal tissues by optimised biochemical methods (mainly enzymatic digestion and/or dissolution in acid), has further promoted its use in composite systems and scaffolds for bone regeneration [8].

In particular, collagen derived from different animal source was observed to present some differences in the aminoacidic sequence, thus influencing the physico-chemical and mechanical properties of the final material. Similarly, the extraction method proved to influence the final properties of collagen molecules and in particular their solubilisation and further ability to crosslink, according to the cleavage sites involved in the process [3,8,61,76]. According to this, the proper selection of the collagen source is fundamental and dependent on the specific final application.

1.3.1 The multi-scale hierarchical structure of Type I Collagen

Collagen is the most abundant insoluble structural protein present in extracellular matrix of mammalian, constituting more than one-third by weight of body protein tissue. About 28 types of collagen can be distinguished and among them, collagen type I represents the most abundant one, especially in tissues like tendons and bones [61,62].

Collagen represents an interesting biomaterial in tissue engineering due to its several properties such as biocompatibility, hydrophilicity, and low antigenicity. Collagen molecules can be divided in several groups, depending on the structure they form; in particular there are only five types of collagen forming fibrillar structures i.e., collagen I,II,III,V and XI, while the others types are characterised by an alternative non-fibrillar organisation, like collagen IV that specifically forms a two-dimensional reticulum [61].

As represented in Figure 1.6, type I collagen can be divided in four main structures, which form a hierarchical configuration responsible for the considerable tensile strength and the stiffness properties of the protein [8,62].

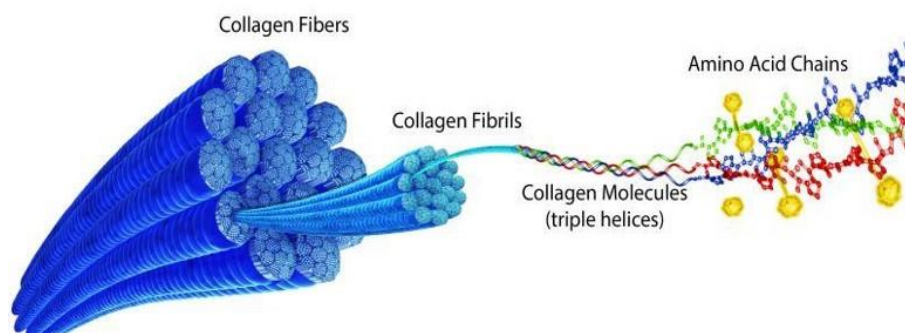


Figure 1. 6 . Multi-scale organization of type I collagen.

The triple-helical structure of collagen arises from the characteristic domain of proline-rich Gly-X-Y polypeptide, which represents the primary structure. In most

of the domains, X is proline and Y is hydroxyproline and each of them has a precise function in order to build the final complex configuration. The secondary structure is given by α -chains, repetitions of the tripeptide (Gly-X-Y) that are linked to each other, building the characteristic triple helix [8]. The short non-helical telopeptides of collagen are covalently crosslinked with other collagen molecules or extracellular matrix molecules. The side chain of glycine, an H atom, is the only one that can fit into the crowded centre of a three-stranded helix, providing the rotation freedom required to form the helix structure [3,8,61].

Hydrogen bonds linking the peptide bond NH of a glycine residue with a peptide carbonyl C=O group in an adjacent polypeptide help to hold the three chains together. The fixed angle of the C-N peptidyl-proline or peptidyl-hydroxyproline bond enable each polypeptide chain to fold into a helix with a geometry such that three polypeptide chains can twist together to form the three-stranded helix tertiary structure or the so-called tropocollagen [61]. The other large variety of amino acids present in the molecule are responsible for the characteristic biochemical and physical features that play a key role in cell attachment and biological functions [8].

The left-handed triple helix, especially in collagen type I, is usually formed by two identical α_1 (I) and α_1 (II) chains and a α_2 (I) chain with about 1000 amino acids and presents a diameter of about 1.5 nm and a length of 300 nm. As shown in Figure 1.7, collagen subjected to electrophoretic denaturation thus present components at different molecular weight and represented by monomeric α -chains (100 kDa), two covalently crosslinked α -chains ($[\alpha_1(I)]_2$) forming dimeric β -components (200 kDa) and trimeric γ -components (300 kDa) formed by three crosslinked α -chains ($[\alpha_1(I)]_2[\alpha_2(I)]$) [3].

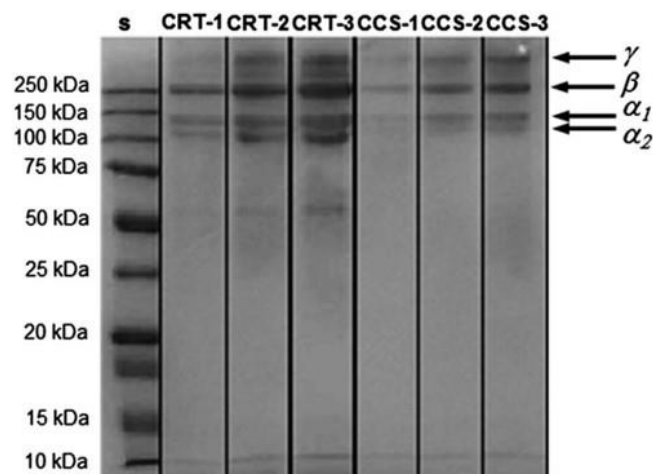


Figure 1. 7 Electrophoretic denaturation of collagen showing monomeric α -chains, dimeric β -components and trimeric γ -components [3].

Thanks to the ability of type I collagen to self-assemble, many triple helix molecules pack together side-by-side, forming collagen fibrils, which represent the quaternary structure [8]. The short segments at either end of the collagen chains are particularly important in the formation of collagen fibrils thanks to the amino acid hydroxylysine which allows to stabilise the side-by-side packing. The molecules organisation provides the main properties to collagen and depends on the type of

tissue considered. Collagen molecules pack to form ordered 67 nm D-banded patterns, that further self-assemble to form micro-fibrils, fibrils and then fibres ranging from micron to millimetres in scale [2,8].

Collagen fibrillogenesis *in vitro* is promoted by exposing collagen solutions at a range of physiological pH (6.5-8), ionic strength and temperature (37 °C), obtaining viscoelastic gels at the macroscopic level [3]. The physiological pH is known to hinder amino group protonation while enhancing lysine reactivity, and together with temperature promote the reconstitution of collagen molecules in structured native-type fibrils [2,3]. During the ordered molecule packing, the amino terminal non-helical fractions are known to induce the initial self-assembly, while the carboxy-terminal non-helical parts trigger the lateral packing of fibrillar units. After neutralisation, collagen systems provide a mesh-like structure, representing a favourable environment for cell seeding and growth [2].

The high biocompatibility and bioactivity of collagen is due to the recognition of the specific aminoacidic sequences for adhesion by cells, that subsequently bind to the protein through integrin receptors. Moreover, the collagenous matrix can be degraded by specific enzymes secreted by cells and replaced by new collagen molecules produced into the intracellular space and further released to the ECM. These mechanisms of collagen removal, remodelling, and replacement are at the basis of the homeostasis of many tissues and organs [8,61,77].

1.3.2 Collagen-based scaffolds

The important role of type I collagen as main component of substrates for cell cultures and tissue regeneration has been recognised since 1950, while most recently, collagen-based scaffolds are increasingly gaining interest as tissue-mimicking constructs in 3D cultures [2,8]. In bone tissue engineering, type I collagen has been processed with different types of techniques to obtain sponges, and membranes to promote tissue regeneration as well as spheres or constructs to modulate the release of drugs and biomolecules [2]. In this scenario, collagen-based scaffolds are mostly reported in form of hydrogels, defined as polymeric matrices able to retain large amounts of water and fluids [61,78,79]. Upon physiological pH and temperature, collagen molecules spontaneously self-assemble *in vitro* to reconstitute solid fibrillar matrices characterised by high water content, mimicking the fibrillogenesis mechanisms occurring *in vivo* [2,8]. The reconstituted matrix provides a cell-friendly 3D environment resembling the properties of the native ECM and thus favouring cell attachment and proliferations. However, commercial collagen solutions used to produce 3D scaffolds are characterised by concentrations ranging from about 2 mg/mL up to 10 mg/mL, resulting in low collagen fibrillar densities (dry-to-wet weight ratio) and poor mechanical strength *in vitro*, thus limiting their use in bone tissue engineering applications [2,80]. Moreover, a significant shrinkage of collagen-based constructs is frequently observed in presence of embedded cells, able to remodel the fibrillar matrix due to its poor visco-elastic properties [2,77].

Therefore, different strategies have been explored to overcome these limitations, that include either the increase of protein concentration before self-assembly or densification techniques, reducing the amount of water after fibril formation, with the final aim to obtain more compact osteoid-like models [2].

Different features can be tailored both at the macro and microscale, acting on the manufacturing processes and regulating the gelation mechanisms, as schematically represented in Figure 1.8 [8].

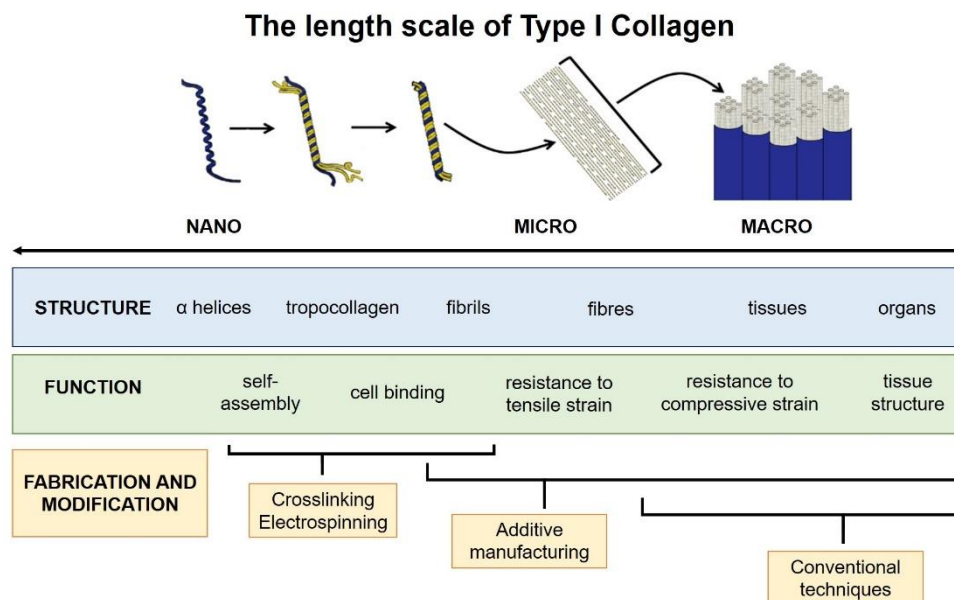


Figure 1. 8 Structure and function of type I collagen at the different length scale and the related possible processes exploited to modify the final properties of the protein.

Denser and stiffer structures have been obtained by reverse dialysis or solvent evaporation of collagen solutions, reaching concentrations up to 100 mg/mL, showing higher proliferation and migration of bone cells [2]. In parallel, plastic compression has been suggested to reduce the excess of liquid component after self-assembly of collagen matrices, leading to preferential orientation of the fibrils and the subsequent evident increase of the scaffold modulus along their direction, as observed by Zitnay and co-workers [81]. Despite the positive biological outcomes, these techniques require long manufacturing times and present significant limits on the final morphological features of the scaffolds. For this reason, they are normally used when studying bone cell activities occurring at the nano-to-microscale without considering higher level of tissue organisation [2,82].

Besides the approaches previously described, different manufacturing processes such as freeze-drying, electrospinning and rapid prototyping techniques have been exploited to produce interconnected porous scaffolds based on collagen able to promote cell migration and fluid diffusion *in vivo* [62]. However, as normally reported for constructs based on natural polymers, collagen scaffolds are characterised by poor stability and mechanical properties, that limit their use in the orthopaedic field [62].

According to the previous observations, strategies to overcome these limitations have been developed, exploiting either crosslinking treatments or the

introduction of reinforcing and stiffer phases into the collagenous matrix, trying to balance mechanical stiffness and adequate structural porosity [8,62].

Composites are normally considered to optimise the performance of materials, combining the advantages of two or more phases. Collagen has been widely incorporated in synthetic polymeric scaffolds in order to improve their biological features and promote cell adhesion while maintaining the mechanical resistance and the 3D structure of the scaffold. Accordingly, Dai and co-workers demonstrated higher production of ECM and glycosaminoglycans (GAGs) with increased concentrations of collagen in poly(L-lactic acid) (PLLA) based scaffolds [83].

Enhanced influence on cell behaviour has been particularly obtained when combining collagen with biologically derived molecules. Collagen/GAGs scaffolds initially designed for skin grafting and further optimised for supporting chondrogenesis and osteogenesis, demonstrated increased cell proliferation and migration throughout the scaffold compared to the collagen matrix alone [62,84]. Moreover, Lee and co-workers developed collagen/chitosan/GAGs scaffolds containing chitosan microspheres for potential drug delivery, observing positive biological response and enhanced mechanical strength due to the electrostatic interactions between the negative charged collagen molecules and the positive charged chitosan [85].

Despite the enhanced performances demonstrated by different studies, the use of synthetic polymers often leads to cytotoxic effects caused by degradation products, while biopolymers and biomolecules have proved to poorly increase the mechanical stability of the resulting scaffolds.

According to the previous observations, one of the most common strategy used in bone tissue engineering, consists in combining soft polymeric matrices, such as collagen, with reinforcing inorganic phases that can provide mechanical support while mimicking the composite nature of natural bone tissue [62]. Besides mineral phases, also gold, silver and carbon nanoparticles have been combined with collagen matrices, where hydrophobic carbon nanotubes proved to increase the mechanical properties and the electrical conductivity of collagen after proper optimisation of the critical phase dispersion and protein interactions. However, the potential toxicity of these particles both at the cellular and systemic level is still a matter of debate [8,59].

Based on their significant biocompatibility, osteoconductivity and bioactivity, bioceramics and bioactive glasses have largely been employed in the regeneration of bone tissue, with an attempt to reproduce the physiological reinforcing mechanism of nano-HA crystals on collagenous and non-collagenous proteins in bone ECM [62]. Calcium phosphates in the form of hydroxyapatite (HA) and tri-calcium phosphate (TCP) are the most commonly reported, due to the demonstrated high compressive modulus of HA and the bioresorbability of most of the TCP phases [62,79]. In parallel, bioactive glasses have been exploited to increase the osteogenic potential of composite scaffolds thank to their high bioactive character, that promotes the deposition of HA nanocrystals in presence of physiological fluids [37,86]. The increase of scaffold stiffness upon addition of such inorganic phases have been often combined to a more positive biological response, where osteogenic

mechanisms proved to be enhanced and accelerated compared to the use of collagen alone [86–88]. Moreover, by exploiting electrostatic bonds or surface functionalisation, calcium phosphates and glass particles have been already combined with therapeutic species and bioactive molecules such as growth factors, showing their potential as delivery platforms [62].

Considering that cell activities are highly affected by biochemical and biomechanical stimuli derived from ECM, collagen-based scaffolds able to provide biochemical signals are gaining large interest in bone regeneration applications. In the clinical practise, collagen sponges loaded with bone morphogenetic proteins (BMPs), as represented in Figure 1.9, are currently used to promote tissue regeneration for both short and long bone fractures and fragment fusion, due to excellent ability of BMPs to induce formation of new bone.



Figure 1. 9 Medtronic's INFUSE bone graft containing BMP-2 used for treating open tibial shaft fractures and promoting spinal fusion [62].

However, due to the rapid degradation kinetics, these scaffolds have been exploited only as delivery platforms, without significantly promoting cell adhesion and migration. At the same time, the rapid or inefficient release of growth factors from the commercially available collagen grafts demonstrated different drawbacks such as the potential damage of surrounded tissues and ectopic bone formation respectively, highlighting the effective need to better modulate and possibly prolong their delivery [4]. Therefore, the use of crosslinking strategies or the combination of collagen with reinforcing inorganic phases, are fundamental factors in the development of bone scaffolds, in order to provide controlled degradation kinetics and to support the regeneration of new functional tissue [8,62].

Taking into account the poor bioactivity of collagen, its combination with bioactive phases is considered a promising strategy to enhance bone formation, increasing mechanical strength and osteogenesis of collagen-based constructs [59].

Hydroxyapatite

Hydroxyapatite (HA) is the main inorganic constituent of human bone, representing about the 65-70% of the total bone mass [89]. In bone tissue, HA crystals grow in gaps present between collagen molecules in form of plates with average length of 50 nm, width of 25 nm and thickness of 2-3 nm and orienting their c-axis parallel to the long axis of fibrils [10]. Collagen has an important role

in the spatial regulation of HA crystals *in vivo*, triggering their nucleation and modulating the mineralisation process, where in the first phase, HA particles grow rapidly in two dimensions, being delimited by the collagen fibrils gap [90]. In this phase, an intense ionic exchange between the core of the forming mineral particle and the surrounding aqueous biological fluids takes place. HA crystals present in the human bone are non-stoichiometric, with a Ca/P ratio of less than 1.67, and show different ion substitutions, where the most abundant one is represented by carbonate ion (CO_3^{2-}) [91,92]. The second stage of the mineralisation process, instead, is characterised by the increase of the mineral particle thickness and the reduction of the ionic exchange. However, thanks to its large specific surface (around $100 \text{ m}^2/\text{g}$), HA remains a metabolically active dynamic entity [93].

Within the biomedical field, synthetic nano-HA has found its principal applications in the bone tissue engineering area thanks to its osteoconductive potential, significantly improving the osteointegration of different inert materials such as metal implants and cements [94–97]. Nano-HA is also particularly attractive as drug carrier due to its solubility in biological fluids and its capacity to penetrate the cell membrane [98], while its high osteogenic potential has been demonstrated by the higher expression of several differentiation markers in osteoblasts (ALP activity, collagen synthesis and calcium deposition), as reported in the study of Jeon and co-workers, where the formation of functional OBs and OCs from human induced pluripotent stem cells (hiPSCs) was observed in HA containing 3D bone scaffolds [99–101].

Looking at the native composition of bone, HA can therefore be introduced in collagen-based hybrid formulations to increase the biomimetic character of the final scaffold, overall favouring higher cell proliferation and differentiation [94,102–106].

The manufacturing of scaffolds combining collagen and HA particles is normally performed by means of direct blending, immersion in simulated body fluids or co-precipitation. The latter is considered particularly promising in mimicking the physiological mineralisation mechanisms, where the deposition of nano-HA crystals is promoted simultaneously to the fibril formation by regulating pH and temperature of collagen aqueous suspensions as well as the content of Ca^{2+} and PO_4^{3-} in solution. The mineralisation of the scaffolds have also been obtained by immersion of the reconstituted collagen constructs in high concentrated calcium and phosphate solutions to promote the nucleation of HA crystals on the previously formed fibrous matrix. However, these methods, together with the normal blending, are often associated to a non homogenous distribution of the HA particles into the organic matrix [107].

When adding previously synthesised HA, the use of nano-sized particles has been preferred to microparticles in order to better increase the compressive stiffness and the biomimicry of the scaffold, while the larger surface area can enhance cell adhesion and the direct chemical bonds with the host tissue [62,107].

A clear evidence of the major osteogenic properties induced by the addition of HA into collagen scaffolds, has been provided by the *in vivo* study of Calabrese et al. [108], who developed a bilayered scaffold: one layer based on type I collagen

and the second layer combining collagen and HA. After four weeks from the implant in mice, the layer containing HA demonstrated faster and complete mineralisation and a greater number of calcium deposits. Moreover, other studies confirmed the improved osteogenesis in presence of Mg and Zn doped HA particles, observing even higher expression of osteogenic factors and the formation of double bone volume compared to the use of non-doped HA rat models [109,110].

Alongside the enhanced osteoconductive and osteoinductive properties, HA containing collagen scaffolds reported evident increase of the mechanical stiffness. However, the degree of increase is significantly dependent from the concentration of both collagen and HA particles as well as the manufacturing process and the crosslinking strategies [107]. In this frame, Liu and co-workers observed that high concentration of HA prevented the reconstitution of the fibrillar structure of silk fibroin scaffold and decrease the final mechanical stiffness due to the poor interfacial adhesion between inorganic and organic phases [111].

As previously mentioned, the design of scaffolds combining collagen and HA mostly reported poor control over the ratio between organic and inorganic phase as well as the final morphological features when exploiting biomimetic *in situ* mineralisation techniques. In parallel, in presence of large amounts of HA, most of the composite constructs are described as highly porous lyophilised matrices or injectable hydrogels [8]. To overcome the morphological limitations presented by conventional techniques, 3D printing technologies are currently under investigation. However, the accurate reproduction of the native composition and structure at the micro and nano-scale still represent a big issue [107].

Bioactive glasses

Similarly to HA, bioactive glasses are classified as materials able to provide an ECM-mimicking surface, thanks to the ability of promoting the creation of a biologically active layer of hydroxyapatite on their surface when in contact with physiological fluids [37,60,112]. Thanks to their superior surface reactivity, bioactive glasses have been reported to show greater bioactivity compared to calcium phosphate bioceramics [60,107]. In addition, the release of calcium and silicon ions as dissolution products have been reported to stimulate osteogenesis by triggering cell proliferation and differentiation by promoting the expression of genes such as RCL and CD44, allowing them to be classified as osteoinductive materials [55,113]. Moreover, some bioactive glasses such as Bioglass 45S5, already approved by the U.S. Food and Drug Administration (FDA), have been demonstrated to trigger not only osteogenic but also angiogenic activity *in vitro* and *in vivo* studies [107].

A higher degree of bioactivity can be obtained using mesoporous bioactive glasses (MBGs) due to their higher surface area and reactivity given by the presence of nanopores [60,114]. MBGs have received considerable attention as multifunctional biomedical devices, due to their ability to synergistically combine an excellent bioactivity with the release of therapeutic agents. In fact, their

extremely high exposed surface area and pore volume allow to load and release active molecules, such as anti-inflammatory or antimicrobial agents. Moreover, the composition of MBGs can be enriched through the incorporation of specific elements (i.e., Sr, Cu) with the aim to combine in a single biomaterial several therapeutic abilities, such as pro-osteogenic, pro-angiogenic and antibacterial properties.

Due to their attractive features MBGs have been used to develop a great variety of devices for tissue engineering, among which hierarchical porous scaffolds with enhanced biocompatibility and osteoconductivity as well as more biomimetic structures to promote a positive crosstalk between material and cells in bone regeneration applications [115,116].

MBGs have already widely proved their great potential in promoting the regeneration of bone tissue thanks to the well-known osteoconductive properties. However, the use of MBGs alone for the design of bone scaffolds has resulted very limited mainly due to the poor mechanical features of the final constructs. The overcoming of these drawbacks has been mostly achieved through the combination of MBGs with other materials and polymers such as type I collagen [53,86]. In addition, their inclusion into the collagen-based composite system is expected to increase the overall bioactive, osteogenic and angiogenic properties of the final bone scaffold, creating a suitable interface between material and cells [86].

Different size, morphology, and composition of bioactive glasses as well as manufacturing technologies have been reported in combination with type I collagen to produce bioactive scaffolds for bone tissue engineering [62,86].

In this frame, the presence of MBG nanofibers proved to limit the shrinkage of freeze-dried collagen scaffolds during the chemical crosslinking treatment and increased the deposition of apatite when soaked in simulated body fluids (SBF), while lowering the final compressive strength [117]. On the contrary, Marelli and co-workers observed that the introduction of nano-sized bioactive glasses increased over 16 times the compressive modulus of the scaffold compared to collagen alone [118]. Moreover, they observed that the ionic dissolution products of bioactive glass particles up-regulated the osteoblastic differentiation, revealing a higher osteogenic gene expression, according to several *in vitro* and *in vivo* studies reported in the literature [86,107,118]. However, in most cases, the reinforcing effect of MBGs as inorganic phase is not enough to increase the mechanical strength and stability of the final scaffolds, reaching values of compressive strength and resorption kinetics still quite far from that of bone natural tissue. Therefore, different strategies have been explored in order to obtain suitable mechanical stability while preserving the advantages of using naturally derived polymers able to stimulate a more positive cell response [8,62,86].

Since the combination of MBGs and collagen does not lead to the spontaneous formation of chemical strong interactions between bioactive glass surfaces and polymeric chains, the resulting composite systems have shown a weak increase of the overall strength compared to the polymeric matrix alone. With the aim to significantly reinforce the final material properties, some studies reported the functionalisation of MBG particles in order to promote chemical bonds between the

organic and inorganic phases [37]. Following this strategy, El-Fiqi and co-workers modified nano-MBGs with amine functionalisation to promote enhanced H-bonding/ionic interactions between the amino-functionalized nanoparticles and the large number of carboxylic groups exposed by the collagen molecules, as schematically represented in Figure 1.10 [119]. The formation of chemical bonds overall promoted a faster matrix gelation related to the self-assembly of collagen molecules and enabled the reconstitution of a stronger material with higher thermal stability.

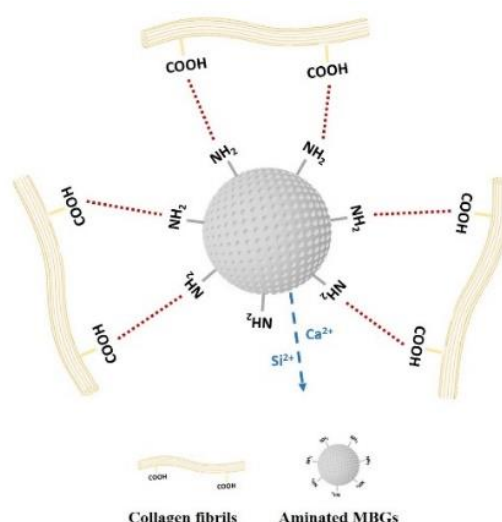


Figure 1. 10 Schematic illustration of chemical interactions between collagen molecules and aminated MBGs.

Alternative routes to increase the final mechanical properties of the composites were focused on the optimisation of the manufacturing process. Indeed, the use of techniques such as lyophilisation or physical compression allows a significant increase in the final strength of the constructs, especially considering natural polymers like collagen. Accordingly, the study of Lee J. H. et al. aimed at investigating the incorporation of nanosized MBG particles into type I collagen hydrogels, further compressed and rolled to increase the final mechanical properties of the hybrid construct, due to the increase in collagen concentration and reduction of the overall amount of water content [120]. The results evidenced a nine-times increase in the storage modulus after the introduction of MBG particles into the collagen matrix and about 150-times increase after the composite matrix compression, highlighting the synergistic influence of MBGs and processing method over the final elastic behaviour of the composite. In addition, the increased mechanical strength of the material induced a faster proliferation and higher expression of osteogenic factors of mesenchymal stem cells (MSCs) cultured on the rolled and compressed collagenous composite matrix.

With the aim to empower the osteogenic potential of bioactive glasses, several studies have reported that the presence of strontium ions beneficially affects cellular behaviour mainly due to its similarity with calcium, enhancing new bone formation while reducing osteoclast resorptive activity [113,121–124]. In fact,

strontium ions are able to activate the calcium sensing receptors on osteoblast surface stimulating the production of osteoprotegerin (OPG), able to bind to the nuclear factor kappa beta ligand (RANKL) responsible for the osteoclastogenesis [125].

The different data present in the literature therefore suggest the high potential of strontium-containing bioactive glasses as pro-osteogenic materials for bone regeneration. However, strategies are required to improve the stability of the systems when combined with natural polymers such as type I collagen [86].

1.3.3 Collagen crosslinking strategies

Despite the combination with inorganic phases is known to provide enhanced mechanical stiffness and stability to collagen-based matrices, by itself, it may be insufficient. In the natural extracellular matrix, neighbouring collagen molecules are covalently bonded through the action of specific enzymes such as lysine hydroxylase and lysyl oxidase that mainly act on lysine and hydroxylysine residues, ensuring the formation of a stable and stiff matrix composed by a dense fibrillar packing, highly resistant to degradation [2,8].

Considering that these mechanisms do not occur *in vitro*, to provide the final constructs with improved mechanical features and slower degradation kinetics, the formation of covalent and non-covalent bonds between protein molecules have been largely explored by exploiting several exogenous biological, chemical, or physical crosslinking methods, of which the most common are summarised in Figure 1.11 [2,8,62,126].

These treatments target the crosslinking between either collagen molecules or the singular three helices, and their efficiency is regulated by different factors such as the stability of the crosslinks, the energy associated as well as the number of residues involved in the treatment [62]. At the same time, the degree of crosslinking can be modulated to precisely tune the final properties of the scaffolds, for instance by controlling the crosslinker infusion to obtain gradients in mechanical properties of collagen hydrogels [2].

In absence of covalent crosslinking, the reconstituted collagen matrices present poor mechanical stability related to a lower protein density and an incomplete molecular crosslinking compared to the native ECM [8].

Based on biomimetic approaches aimed at reproducing the crosslinking mechanisms *in vivo*, the extent of inter-molecular bonds induced by enzymes such as microbial transglutaminase or non-enzymatic glycosylation (glycation) has been explored on collagen matrices, resulting in high cytocompatibility of the final construct but only slight enhancement of the matrix strength, insufficient for supporting bone tissue regeneration [8,62].

Among the chemical agents, glutaraldehyde (GTA) has been widely reported as the most used and effective crosslinker of collagen and biological tissues, thanks to its high reactivity with several functional groups present on proteins. Moreover, at high concentrations, GTA has demonstrated to form long polymeric chains, that promote the bond of amino residues located far apart, therefore enhancing the extent

of crosslink formation. Despite the significant stability and strength obtained on collagen-based matrices, GTA has been often associated with cytotoxic effects both *in vitro* and *in vivo*, due to the presence of residual aldehydes non-specifically linked to the matrix that are released during its degradation [8,62].

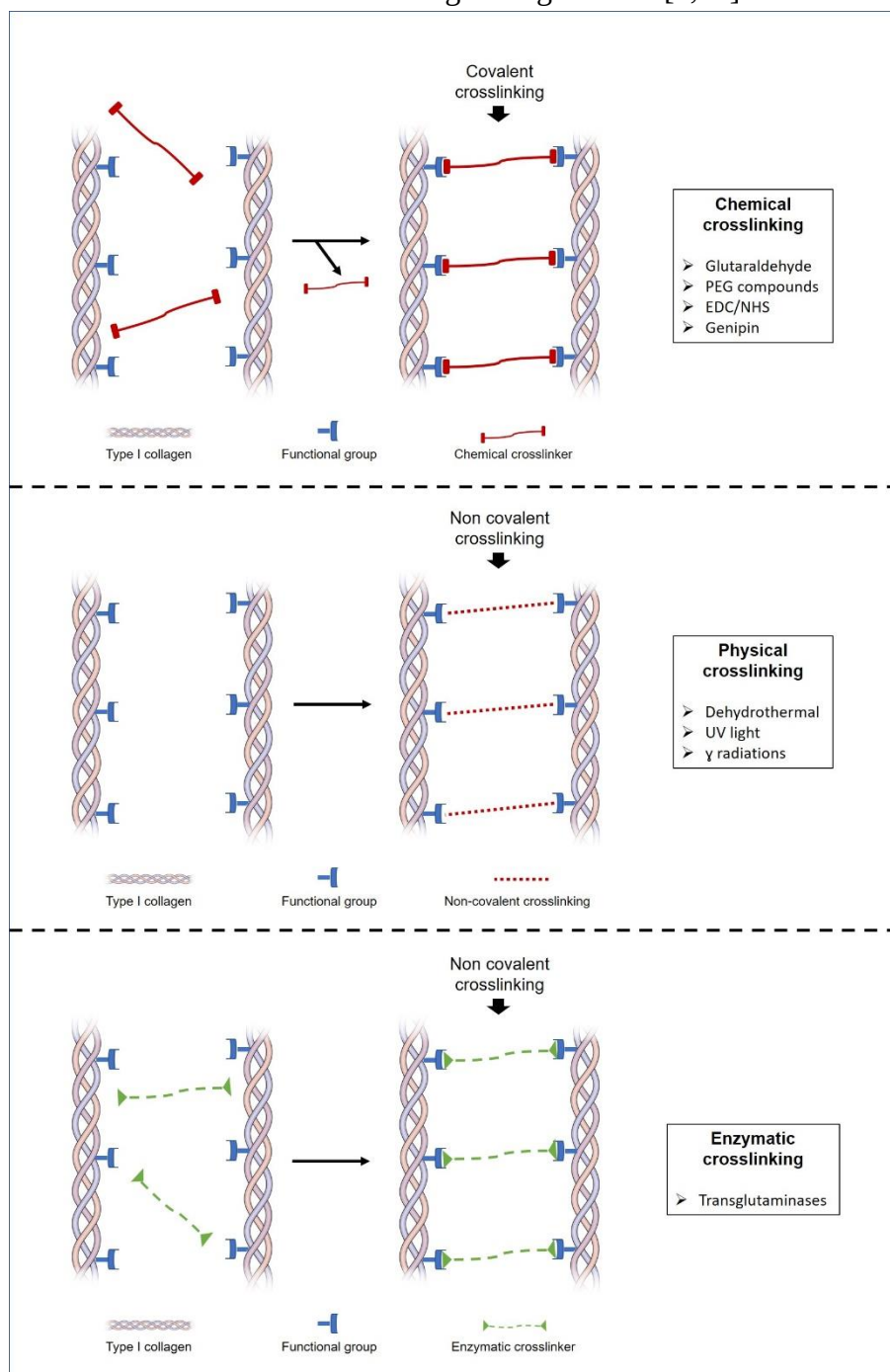


Figure 1. 11 Schematic illustration of the most common chemical, physical, and enzymatic crosslinking methods used on collagen-based materials.

As alternative chemical crosslinkers, carbodiimides have often been exploited to crosslink collagen molecules and promote their functionalisation with other biomolecules. Carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) are known to be zero-length crosslinkers and form covalent bonds between carboxyl and amino groups of collagen molecules,

where water-soluble urea represents the only by-product of the crosslinking reactions and can be easily removed to limit potential cytotoxicity. However, the crosslinking efficiency of carbodiimides is significantly limited due to their inability to polymerise in addition to the formation of zero-length covalent bonds, that hinder the bridging between distant amino acidic residuals or adjacent collagen fibrils [62]. According to this, several studies in the literature reported contrasting results about the effective increase of mechanical properties of collagen-based scaffolds after EDC treatments, where some studies such as the one reported by Kwangwoo et al., suggested strategies to increase the degree of crosslinking by modulating process time and temperature as well as the use of ethanol instead of water-based solvents [62,127].

A good balance between crosslinking efficiency and cytocompatibility of the process was extensively reported using genipin, a natural plant-derived chemical crosslinker. As most of the chemical treatments, the extent of crosslinking can be controlled by optimising parameters such as time, pH, and concentration according to the final applications. Genipin-crosslinked scaffolds proved to induce a higher increase of mechanical strength and resistance at both hydrolytic and enzymatic degradation, compared to the more common treatments with GTA and EDC. However, the potential cytotoxicity of the natural agent and the alteration of the morphological features of scaffolds observed in some studies, are still matter of debate [8].

Despite the limited data reported in the literature, multi-functional poly-ethylene glycol (PEG) systems obtained extensive interest in the regenerative medicine field to either promote crosslinking of molecules or their functionalisation, and their use proved the effective stabilization of tissue grafts and collagen hydrogels. However, it is still unclear whether such agents can result in analogous stability to traditional crosslinking methods, without any cytotoxicity [128].

Besides the use of chemical agents, physical crosslinking approaches consider the use of thermal treatments such as the dehydrothermal method or exposure to radiant energy such as UV light and gamma radiations [8].

The hydrothermal method exploits high temperatures and vacuum to remove the water content from collagen scaffolds while promoting intermolecular crosslinking between carboxyl and amino groups. Despite the use of this physical treatment has been largely reported also for the sterilisation of the constructs, the long duration and the high temperatures involved in the process, reported partial collagen denaturation and the subsequent decrease of the resistance to enzymatic degradation [8,62].

Similar observations about the potential denaturation of the protein following the crosslinking process, were reported for UV curing treatments on collagen scaffolds. In this case, the prolonged exposure to UV light lead to the formation of free radicals, predominantly on aromatic residues of tyrosine and phenylalanine, that can react together especially in presence of photosensitizers such as riboflavin. Moreover, when the constructs are exposed to the specific radiation, the

crosslinking reactions mostly concentrate at the surface, resulting in non-homogeneous mechanical properties of the scaffolds [62,129].

To overcome these limitations, the functionalisation of collagen molecules to promote photo-crosslinking mechanisms have been recently reported as promising strategy to enhance the final properties of collagen-based constructs.

As reported by Tronci and co-workers, the hierarchical structure of collagen represents a stringent constraint compared to other linear polymers, since the molecule functionalisation could alter its structural and physico-chemical properties, thus compromising the final solubility and biofunctionality [3].

It has been observed that the introduction of aromatic bulky groups is responsible for the formation of a stiffer material that can influence the final architecture and mechanical properties of the scaffolds. These observations can be explained by the inability of aromatic residues in forming hydrogen bonds in contrast to lysine terminations on collagen molecules, that are able to stabilise the triple helical structure bonding to polyproline residues. Even if the formation of hydrophobic and electrostatic interactions between aromatic moieties can alter the native conformation of the protein, it has been reported to increase its thermal stability [3]. These observations therefore suggest that the final physico-chemical and mechanical properties of the collagen-based scaffolds are significantly influenced by the moieties selected to functionalise the protein and can be exploited to tune the final performances of the constructs.

In this frame, the methacrylation of native collagen molecules have been investigated in different studies, to induce the rapid photocuring of collagen solution in presence of photoinitiators and upon UV exposure, as illustrated in Figure 1.12.

Different studies reported that the UV curing of methacrylated collagen proved a superior and faster increase of the mechanical properties of the final matrix compared to other crosslinking methods. In particular, a temperature-dependent reversible self-assembly was observed, whereas the triple helical conformation and the native physico-chemical properties of the protein proved to be preserved [3,130,131].

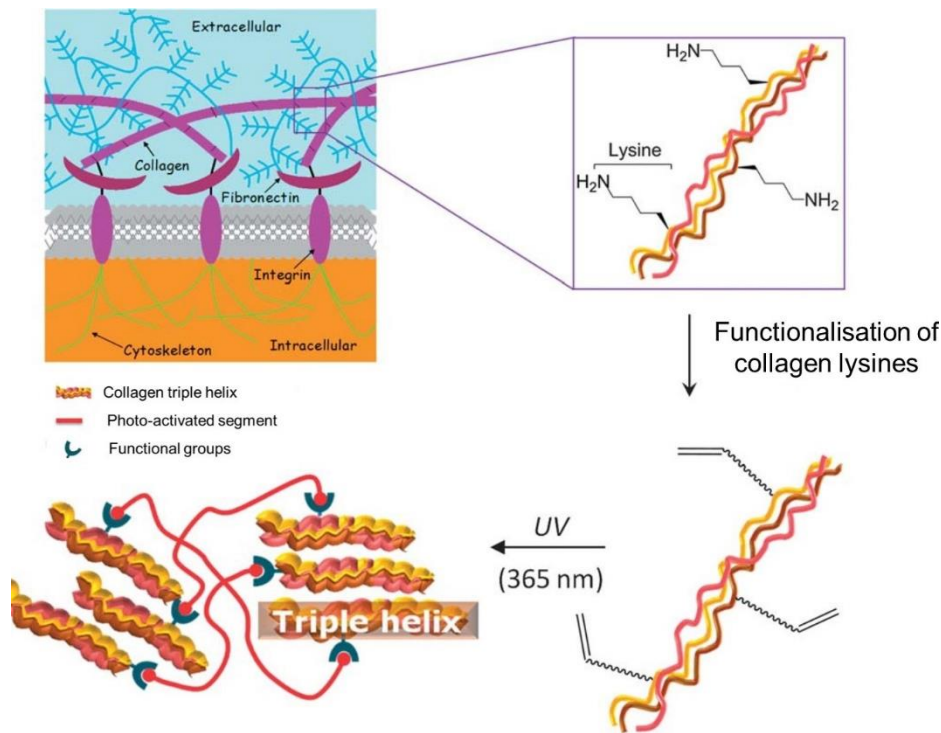


Figure 1. 12 Image showing the functionalisation of the native collagen molecules with photo-active compounds and the subsequent crosslinking in presence of cytocompatible photoinitiator and upon exposure to UV light [3].

Despite different strategies resulted to be promising in terms of increasing of scaffold mechanical properties (e.g., tensile strength and modulus), a gold standard crosslinking method for collagen-based constructs has not been defined yet. Furthermore, different treatments were observed to compromise the final biocompatibility of the systems, hindering the cell attachment and infiltrations upon alteration of the surface biochemistry as well as the presence of crosslinker residuals [2].

The combination of multiple crosslinking strategies and the introduction of secondary inorganic phases have therefore been suggested to increase the final stability of the scaffolds [62].

1.4 Fabrication of bone scaffolds towards biomimetic structures

The introduction of bioactive inorganic phases into polymeric matrices, especially in the case of natural polymers, represents a key factor in the design of 3D constructs suitable for bone tissue engineering able to better mimic the composite nature of native tissue while ensuring increased mechanical properties and load distribution. Moreover, the possibility of combining multiple stimuli, such as chemical, structural, topographical, and biological cues, has shown to be a very promising strategy in the regeneration process by guiding specific cell responses [5,6].

The complex and unique 3D microenvironment of bone tissue is well-known to orchestrate cell activity and cross-talk, fundamental for its morphogenesis and

maintenance [132]. Besides the control at a macroscopic level, the structural features at the micro- and nano-scale can regulate osteogenesis and angiogenesis, guiding cell adhesion, proliferation and differentiation [1].

Conventional techniques used for the fabrication of bone scaffolds are based on subtractive approaches, where the control over the final shape, porosity and interconnectivity is significantly variable and limited [1,5,40,80]. With respect to collagen-based materials, the most common manufacturing approaches concerns the extrusion of single fibres, the formation of cylindrical strands and films in addition to porous sponges with different shapes obtained after the freeze-drying of mould casted constructs [80]. Despite the reported techniques enable the potential combination of collagen with additional polymeric and inorganic phases, the restrictions on the final morphological features are evident.

With these perspectives, additive manufacturing (AM) and electrospinning technologies, are currently attracting considerable efforts for the design of biomimetic scaffolds able to reproduce the natural features of healthy bone in several respects [40].

Electrospinning has often been considered to produce sub-micrometric fibrous matrices highly resembling the natural ECM, demonstrating its higher potential to design biomimetic bone scaffolds even if it is classified among the conventional manufacturing approaches [133].

In contrast to all the previously cited techniques, additive manufacturing is the only approach that enables the fabrication of 3D scaffolds with highly controlled inner structure and pore morphology, while providing higher reproducible, time-saving and cytocompatible conditions [58].

Despite the use of these technologies dates back to more than two decades, the detail and precision level required to design complex constructs has only been recently developed [58]. Moreover, a wider range of synthetic and natural derived materials has been optimised to be processed with these techniques obtaining high accuracy of the final constructs [58].

More recently, the design of biomimetic constructs containing both cells and biochemical cues have been suggested to improve the regenerative potential of bone scaffolds, where the use of AM technologies allows the realisation of living biomaterial constructs and smart delivery platforms [5]. The biofabrication of bone tissue thus represents a cutting-edge strategy able to control not only the morphological features of scaffolds at multiple levels, but also the spatial localisation of different cells and biomolecules, whose interactions can boost the tissue regenerative process mimicking bone physiology [5]. Considering the metabolically active nature of bone, strategies involving the combination of different cell types such as bone marrow stromal cells and endothelial cells, as well as the use of bioreactors to promote the correct perfusion and mechanical stimulation of the scaffolds, have gained increasing interest and showed great ability in enhancing osteogenesis and angiogenesis [5].

Despite AM and electrospinning technologies proved their higher potential in the realisation of biomimetic bone scaffolds, the choice of biomaterials is still limited, particularly in the case of naturally derived materials and biopolymers such

as type I collagen. These materials must be treated in a narrow range of thermal and chemical conditions to preserve their native physico-chemical properties and therefore restrict the spectrum of the suitable manufacturing techniques, sometimes requiring proper process modifications to guarantee the final processability [5,134,135].

1.4.1 Additive manufacturing technologies

The introduction of additive manufacturing technologies, also known as rapid prototyping, in the field of bone scaffold manufacturing has significantly helped to overcome most of the limitations linked to the final architectural features as well as the use of harsh and toxic solvents required in some conventional techniques.

The additive manufacturing approach is based on the layer-by-layer reproduction of 3D models previously obtained either by using computer aided design (CAD) methods, or exploiting the data contained in medical images derived from 3D scan of specific tissue portions [1]. As schematically illustrated in Figure 1.13, the selected digital models are then converted into STL files that describe the three-dimensional pattern as overall assembly of 2D horizontal plans, where each layer is closely connected to the previous one to create the final solid construct [40].

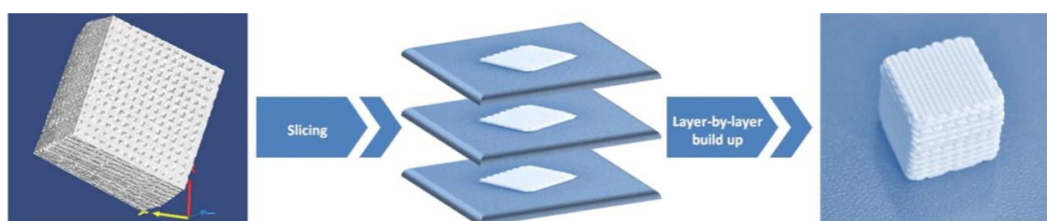


Figure 1. 13 Image illustrating the main steps of rapid prototyping: the original CAD model is processed by means of specific software and sliced into horizontal layers, that are subsequently used as instructions to build layer by layer the final 3D scaffolds.

Since 1990s, rapid prototyping technologies have been largely introduced in the medical field demonstrating enhanced efficiency, speed and customisation compared to the other conventional methods, providing the opportunity to tailor the final construct properties to match patient's specific requirements [1]. The physico-chemical properties of the scaffolds can vary among the structure due to the potential combination of multiple materials and biomolecules that can impart specific engineered signals. The opportunity to create differences in spatial distribution and size of pores, mechanical and chemical features, therefore represents a key factor in the development of constructs mimicking the complex composition and architecture of the bone matrix [5,58].

Among the wide spectrum of AM technologies available for bone tissue engineering, the selection of the specific technique is significantly constrained by the nature of the chosen starting materials and determines the final features and resolution of the scaffold [58]. In this scenario, stereolithography (SL) techniques enable to reach high resolution of the final constructs mainly reducing the spot of the UV laser used to induce the solidification of photocurable resins. Similarly, the selective laser sintering (SLS) approach exploits high power lasers such as carbon

dioxide, to bind together the material powders, where higher precisions can be achieved by reducing the material layer thickness as well as the particle size. On the contrary, in three-dimensional printing (3DP) techniques, powders are assembled by using liquid droplets of bonding agents deposited by inkjet printheads, where the final scaffold is subsequently obtained after removing the unbonded powders. Moreover, 3DP processes performed at room temperature have been optimised to favour the use of a wider range of material powders and the potential combination with biomolecules such as proteins and growth factors for the creation of more biologically active constructs [40].

Considering a different approach, the fused deposition modelling (FDM), represents the most commonly used and versatile technique to produce bone scaffolds, and is based on the extrusion of molten thermoplastic materials and composites from nozzles of different sizes that regulate the final dimension and resolution of the constructs [4,58]. All the extrusion-based printing techniques, that use different polymeric and composite materials, have subsequently been developed based on the principles of FDM approach.

All the reported techniques have been largely used to produce bone scaffolds for both *in vitro* and *in vivo* applications, where different studies reported satisfactory results in terms of cell viability and proliferation. On the contrary, adverse cytotoxic effects linked to the potential presence of binder residuals as well as the lower bioactivity of the starting materials have been detected [4,5].

In particular, the characteristic process conditions of AM technologies such as SL, SLS, 3DP and FDM significantly limit the range of scaffold building materials, mostly hindering the use of biopolymers such as type I collagen and their composites [40]. Moreover, all these methods lack the ability to design constructs containing controlled distributions of viable cells and thus the fabrication of ECM-mimicking scaffolds [5,40].

Bioprinting

The necessity of finding more sophisticated approaches enabling the reproduction of complex architectural features while combining ECM-derived materials, cells, and biochemical cues, led to the identification of a narrower group of AM technologies, classified as bioprinting techniques [5,113]. These methods aim at overcoming the limitations presented by the other AM approaches to design biomimetic constructs, enabling the use of biomaterials containing cells, defined as bioinks, and their controlled distribution into the printed structure [135].

As a matter of fact, bioprinting techniques exploit either mild or ambient processing conditions while reducing the exposure to external sources and stresses that can hinder the viability of cellular components. Despite these techniques have been specifically designed to enable the processing of inks containing cells and biological molecules, their use has often been reported also with cell-free biocompatible materials, defined as biomaterial inks. Regardless of the presence of

cells and bioactive molecules, hydrogels represent the most used biomaterials in the additive manufacturing of biomimetic scaffolds [5,136].

Up to now, the biofabrication of bone tissue has been explored mainly using laser-assisted, inkjet-based and extrusion-based technologies, as represented in Figure 1.14.

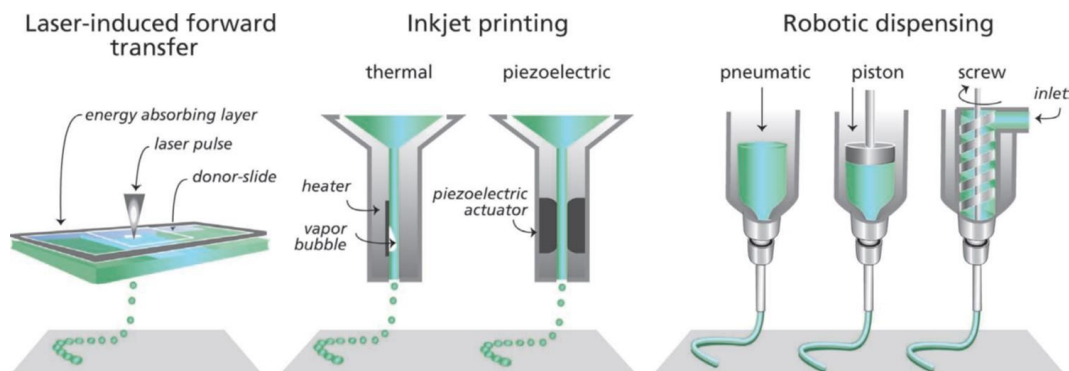


Figure 1. 14 Schematic representation of the main bioprinting approaches used to design bone-like scaffolds.

In laser-assisted bioprinting, a pulsed laser source is used to induce the deposition of small bioink droplets from receiving substrates onto solid-surfaces. The target, transparent to the laser wavelength, is connected to the biomaterial substrate via a laser-absorbing interlayer generating the high-pressure bubbles responsible for the bioink droplet release. This nozzle free technique enables the processing of materials in a wide range of viscosities (about 1-300 mPa.s) while avoiding potential issues linked to the nozzle clogging. Moreover, its first application reported the successful printing of a nano-HA based scaffold directly into mice calvaria defects, demonstrating the feasibility of *in vivo* bioprinting and its great potential in future clinical applications. Despite the promising results and the high resolution obtained, laser assisted printing requires long manufacturing times for the realisation of larger constructs, limiting the final size of the scaffold. Moreover lower cell viability has been demonstrated compared to the other bioprinting approaches [5,40,135,137].

On the contrary, higher printing speeds, enhanced cell viability and lower costs have been demonstrated for ink-jet technologies, characterised by the dispersing of picolitre bioink droplets through a small orifice, obtaining the precise deposition of small material volumes and thus promoting high resolution of the final constructs. The formation of droplets can be induced by either piezoelectric or thermal mechanisms, that are often reported to create local high stresses able to damage the integrity of cell membranes. The main disadvantages of these techniques rely in the frequent nozzle clogging and the extremely low viscosities (< 0.1 Pa.s) required for the material dispensing. Similarly to laser-based techniques, inkjet bioprinting is therefore exploited for the realisation of high resolution scaffolds with limited size [58,135,138].

With the aim to widen the range of processable biomaterials while enabling the realisation of scaffold with larger dimensions, extrusion-based technologies, also reported as robotic dispensing, result the most used, promising, and versatile

techniques used in bioprinting of bone scaffolds. The process foresees the continuous dispensing of cell and material filaments through micro-sized nozzles by exploiting pneumatic, piston- or screw-driven extrusion mechanisms. Based on the delay of the compressed air observed in pneumatic printheads, piston-driven systems are normally preferred to achieve a higher control over the material flow from the nozzle. At the same time, the use of screw-based systems has been indicated for favouring the extrusion of bioinks characterised by higher viscosities, even though larger pressure drops at the nozzle have been evidenced [5,40,137]. These techniques demonstrated the potential realisation of clinically relevant size constructs with suitable timing. However, compared to laser-induced and inkjet bioprinting, the final resolution of the scaffolds is significantly lower and is highly dependent on the specific rheological properties of the selected materials.

Regardless of the considered bioprinting approach, the final shape fidelity and printing resolution are strongly influenced by the visco-elastic properties of the developed bioinks or biomaterial inks. As far as extrusion-based printing is concerned, specific rheological properties such as shear-thinning and fast shear recovery are required in order to enable the proper extrusion as well as the stabilisation of the post-printed structures [139]. Similarly, the high resolution obtained by laser-induced and ink-jet techniques can be highly compromised if the fast gelation and crosslinking of the deposited material is not guaranteed [5]. According to this, several approaches have been explored to increase the processability and the stabilisation of the printed constructs upon deposition, according to the specific physico-chemical properties of processed materials [135,140,141].

Based on the previous observations, the widely reported poor visco-elastic properties and slow gelation mechanisms of collagen, significantly hinder its use for the additive manufacturing of bone scaffolds. However, despite the potential issues derived by its soft nature, collagen still represents a promising tool for the bioprinting of ECM-mimicking bone scaffolds and recently, different strategies have been explored to improve its printability [142].

Considering the low viscosities of collagen solutions, most of the 3D printed constructs have been developed exploiting low temperatures with the aim to increase the material viscosity in order to deposit solid frameworks. Different porous constructs were therefore realised performing a cryogenic plotting, that foresees the deposition of material strands at temperatures lower than 0 °C, to induce the direct freezing of the structure and its subsequent lyophilisation [143,144]. However, this approach has shown to compromise the simultaneous printing of materials and cells, thus hindering the design of scaffolds with advanced functions. In parallel, the additional freeze-drying process is reported to alter the resulting porosity and morphology of the scaffold if not adequately controlled [142,145].

Enhanced visco-elastic properties and higher stabilisation of the deposited constructs have been reported by either increasing the collagen concentration or blending with synthetic polymers. In this frame, commercially available products are therefore based on highly concentrated collagen solutions characterised by

evident shear thinning and increased material strength upon reconstitution, alongside with superior cytocompatibility and cellular remodelling ability compared to synthetic polymers [142].

However, the extrusion of high concentration collagen solutions still results in the design of constructs characterised by low precisions and simple architectures [79].

Most recently, the manufacturing of higher complex constructs based on collagen was explored by exploiting the extrusion into supporting baths able to sustain the deposition of the soft biopolymer. The reconstitution of a solid matrix was subsequently achieved at physiological temperature, enabling the removal of the supporting material in the liquid state, as represented in Figure 1.15 [140,141,146]. This innovative approach, known as freeform reversible embedding of suspended hydrogels (FRESH), was exploited by Lee and co-workers to realise human heart components at various scales and characterised by up to 20-micron filament resolution. In detail, the optimisation of the physico-chemical properties of the supporting bath enabled the extrusion of high concentrated collagen bioinks, also favouring a previous stabilisation of the deposited strands exploiting the acidic pH of collagen solutions and the buffering ability of the supporting material [146].

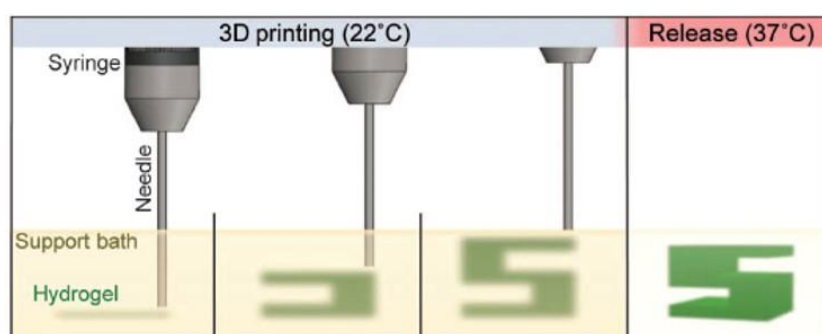


Figure 1. 15 Schematic illustration of the freeform reversible embedding of suspended hydrogel (FRESH) approach used for the extrusion printing of low viscosity biomaterials [140].

The resolution of the final construct can be additionally regulated by the proper selection of the printing nozzle. However, smaller diameters are responsible for higher shear stresses that can significantly hinder cell viability when using bioinks. At the same time, the processing of composite biomaterial inks combining polymeric and inorganic phases still represent an issue, where high concentrations of inorganic phase and potential material aggregation can hinder the proper extrusion especially when using smaller needle diameters [5,135,138,139]. Therefore, the realisation of complex architectures based on collagen-based composite biomaterials for the design of biomimetic bone scaffolds still requires further optimisation of the described techniques.

1.4.2 Electrospinning technologies

For decades electrospinning has been used to obtain sub-micrometric polymer fibres, and more recently, has gained large interest for the fabrication of mesh-like

biomaterials, able to mimic the complex nanostructure of native ECM [8]. This manufacturing process exploits the application of high electric fields that trigger the formation of charged fine material filaments, subsequently deposited on a grounded collector in dry state upon solvent evaporation. Polymeric melts or solutions are normally extruded through metallic needles by regulating the slow material flow by means of syringe pumps. At the same time, the high-voltage power supply is responsible for the creation of an electric field that guides the material jet, created when the electric forces overcome the surface tension of the material, towards the depositing collector [133,147].

According to the process set-up and the physico-chemical properties of the material, polymeric fibres with diameters ranging between tens to hundreds of nanometres can be obtained. The resulting nanofibrous architectures are characterised by high surface area, that can be exploited to adsorb biological molecules and aminoacidic sequences useful to increase cell adherence [40]. In this frame, Li and co-workers proved the successful controlled release of bioactive species such as BMP-2 and dexamethasone, incorporated in serum albumin nanoparticles and subsequently electrospun with a polymeric blend of PCL and PEG, where the implantation of the developed matrix in rat calvarian defects resulted in a significant degree of tissue repair [148].

Alongside the specific properties of the material and the process parameters, the selection of the collector specifically determines the morphological features of the resulting matrix. The most common configuration considers the use of a flat plate collector to obtain randomly oriented fibre mats. On the contrary, as represented in Figure 1.16, rotating drum collectors and mandrels have been used to obtain aligned fibre mats or tubular structures, respectively [40,149,150].

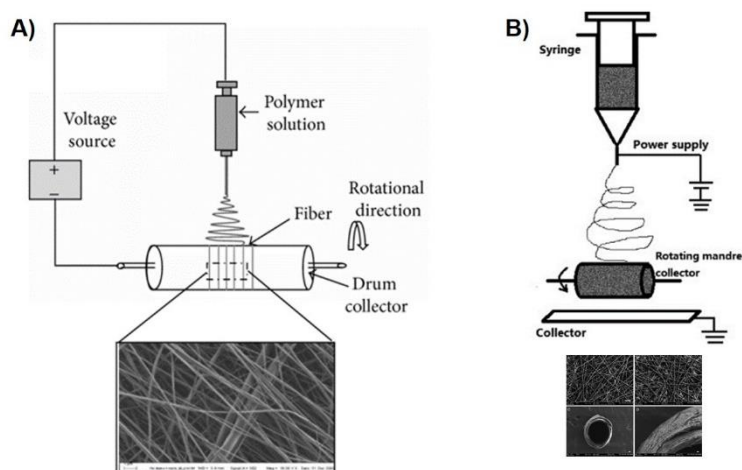


Figure 1. 16 Electrospinning setup implemented with a drum collector (A) and a rotating mandrel (B), to obtain aligned fibres and tubular structures, respectively.

More recently, direct writing electrospinning of polymeric solutions has been exploited to fabricate electrospun scaffolds characterised by regular and more complex geometries, in an effort to simulate the outcomes obtained by additive manufacturing technologies. However, a limited range of synthetic materials has

currently proven an adequate processability with this technique, where the physico-chemical properties of the material and the required short distances between the spinneret and collector often hamper the achievement of well-defined and regular scaffolds [147,149].

In contrast to additive manufacturing technologies, electrospinning often requires the use of harsh and toxic solvents to increase the volatility of the processed material and promote the proper solvent evaporation during the deposition step [8]. Unfortunately, the high toxicity and the salt content of most of the conventionally used solvents hampered the final cytocompatibility of the electrospun constructs [133,151].

In particular, these aspects have been reported to compromise the processability of naturally derived polymers such as collagen with electrospinning technologies. As demonstrated by Zeugolis et al. [151], the harsh solvents and process conditions can potentially alter the conformation of collagen peptides while preventing the natural self-assembly, mainly leading to protein denaturation. Accordingly, several strategies exploiting the use of relatively benign solvents are currently under investigations with the aim to preserve the native features of collagen while guaranteeing high cytocompatibility of the constructs [151–153]. In this frame, few studies reported the use of ethanol and water-based solutions to produce collagen electrospun scaffolds, exploiting the subsequent chemical crosslinking to increase the mechanical strength and the stability in aqueous medium. Despite the achievement of sub-micrometre fibrous mats, the morphological features were partially lost after the chemical treatment, while no specific evidence about the preservation of collagen native properties were provided.

Moreover, most of the studies reported in the literature demonstrated that the combination with synthetic polymers such as polyethylene oxide (PEO), polyurethanes (PU) and polycaprolactone (PCL) can facilitate the processing of collagen, increasing the stability of the deposited fibrous matrix [40,149,150]. In this frame, Yu and co-workers reported the good stability and tailored degradation rate of electrospun nerve guides constituted by collagen and PCL, observing high cell proliferation *in vitro* and the effective sciatic nerve regeneration in rats [150].

Despite the addition of collagen proved to increase cell adhesion compared to purely synthetic substrates, the partial loss of its functionality and molecular structure was documented by several studies [8].

Up to now, the use of electrospun scaffolds based on collagen has found limited application in bone tissue regeneration, where it has been mainly combined with synthetic polymers to create fibrous mats for the controlled release of pro-osteogenic agents [4]. However, recent studies reported the promising combination of collagen with hydroxyapatite particles to form biomimetic fibrous composite systems able to enhance the osteogenic response *in vitro*, laying the foundation for further investigation [153,154].

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CHAPTER 2

Materials and methods

2.1 Introduction

Part of the data reported in this chapter have been previously published [1–5].

The aim of this research was the development and optimisation of hybrid bioactive formulations based on Type I collagen, strontium containing mesoporous bioactive glasses (Sr-MBGs) and nano-hydroxyapatite (nano-HA), suitable for the design of bone scaffolds by means of 3D extrusion printing and electrospinning technologies. Type I collagen and nano-HA represent the main organic and inorganic phases of bone respectively, while Sr-MBGs were used to improve the potential bioactive and osteogenic features of the composite material. As illustrated in Figure 2.1, a comprehensive characterisation of the developed materials was conducted to enable the proper optimisation of the printing process and the design of high-resolution scaffolds. The chemical crosslinking of the materials was further investigated with the aim of maintaining the overall cytocompatibility of the system as well as the release of strontium ions.

In addition, collagen-based systems suitable for the design of biomimetic scaffolds by means of electrospinning technologies were developed after the proper optimisation of solvents, polymeric concentrations, and process parameters.

In this chapter, the synthesis and characterisation of the hybrid formulations are described alongside the optimisation of the printing process according to the properties of the systems.

In detail, in Section 2.2 are reported all the protocols used for the synthesis of the hybrid formulations and the different materials used for the printing process as well as the chemical crosslinking strategies explored (Section 2.2.5).

The following sections are dedicated to the different methods used to investigate the morphological, physico-chemical, and bioactive properties as well as the ion release kinetics of the systems before and after the chemical crosslinking (Section 2.2.6 – 2.2.10).

A large part of the chapter (Section 2.2.11) is dedicated to the rheological assessment that enabled the investigation of the visco-elastic properties of the hybrid formulations before and after the sol-gel transition, followed by the printing tests performed using a commercially available 3D Bioprinter (Section 2.2.12).

The formulation of the collagenous systems for electrospinning applications is described in Section 2.3, alongside the study of the process parameters to obtain submicrometric fibrous scaffolds, as schematically represented in Figure 2.1.

Finally, the biological assessment performed on the different systems developed for the extrusion printing of bone scaffolds is presented in Section 2.4.

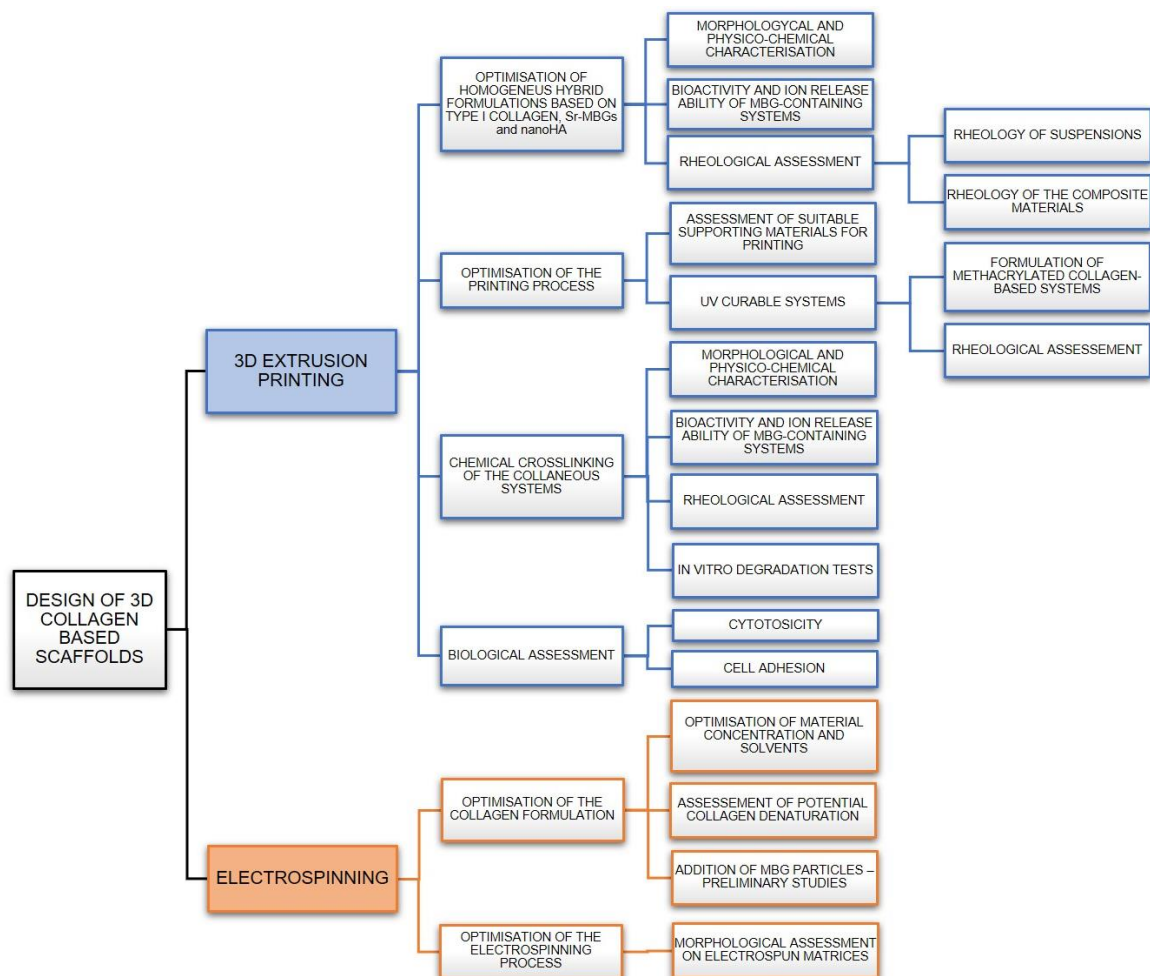


Figure 2. 1 Scheme of the study to produce 3D collagen-based scaffolds by means of extrusion printing or electrospinning technologies.

2.2 Design and characterisation of formulations for 3D extrusion printing of bone scaffolds

Part of the work described in this section has been previously published [1–3].

This section is dedicated to the design of the collagen-based hybrid formulations for the 3D extrusion printing technologies alongside the strategies explored to obtain biomimetic and bioactive bone scaffolds. The different subsections describe in detail the approaches used for the material characterisation, the printability assessment and the different crosslinking methods explored.

2.2.1 Synthesis of the inorganic phases

Micro- and nano-sized MBGs containing 4% molar strontium were synthesised exploiting two different methods and following the protocols developed and optimised based on previous studies of the IRIS research group [6,7]. The incorporation of different concentrations of strontium (1%, 2%, 4% molar) in the glass formulation was previously studied by the IRIS group and the 4% molar concentration was selected for this study in order to maximise the total amount of ion released while ensuring no cytotoxic effects [7,8].

The synthesis of nano-HA particles was optimised exploiting a hydrothermal method and the use of a cytocompatible dispersing agent (Darvan 821-A) to produce biomimetic rod-like particles, as published in our work [3].

Synthesis of Sr-containing MBGs by the Aerosol Spray-Assisted Approach (MBG_SD_Sr4%)

Mesoporous bioactive glass microspheres with 4% molar percentage of strontium ($\text{Sr/Ca/Si} = 4/11/85$) were synthesised by the aerosol spray-assisted approach and are hereinafter mentioned as MBG_SD_Sr4% [1,7]. In brief, a pre-hydrolysed TEOS solution was added drop by drop to an aqueous solution of Pluronic P123 (EO20PO70EO20, average $M_n \sim 5800$, Sigma Aldrich, Milan, Italy) and kept stirring for about 1 h. The calculated amount of calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99%, Sigma Aldrich, Milan, Italy) and strontium chloride (SrCl_2 , Sigma Aldrich, Milan, Italy) were subsequently added and the final solution was sprayed (Büchi, Mini Spray-Dryer B-290, Büchi Labortechnik AG, Flawil, Switzerland) using nitrogen as the atomizing gas. The resulting powders were collected and calcined at 600 °C in air for 5 h using a furnace (Carbolite 1300 CWF 15/5, Carbolite LTD, Hope Valley, United Kingdom).

Synthesis of Sr-containing MBGs by a traditional base-catalysed sol-gel method (MBG_SG_Sr4%)

Nano-sized mesoporous bioactive glasses containing 4% molar percentage of strontium ($\text{Sr/Ca/Si} = 4/11/85$) were synthesized exploiting a base-catalysed sol-gel method and are hereinafter mentioned as MBG_SG_Sr4% [2,7]. Briefly, Sr-containing nanosized MBGs were produced dissolving 6.6 g of cetyltrimethylammonium bromide (CTAB, $\geq 98\%$, Sigma Aldrich, Milan, Italy) and 12 mL NH_4OH (ammonium hydroxide solution, Sigma Aldrich, Milan, Italy) in 600 mL of ddH₂O. 30 mL of tetraethyl orthosilicate (TEOS, Tetraethyl orthosilicate, Sigma Aldrich, Milan, Italy), 4.13 g of calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99%, Sigma Aldrich, Milan, Italy) and 1.68 g of strontium chloride hexahydrate ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, for analysis EMSURE® ACS) were subsequently added and the resulting suspension was kept under vigorous stirring for 3 h. After centrifugation (Hermle Labortechnik Z326, Hermle LaborTechnik GmbH, Wehingen, Germany) at 10,000 rpm for 5 min, the final precipitate was washed once with distilled water and two times with absolute ethanol, and finally dried at 70 °C for 12 h. The powders were further calcined at 600 °C in a furnace

(Carbolite 1300 CWF 15/5 Carbolite Ltd., Parson Lane, Hope, Hope Valley, UK) to avoid CTAB residues.

Synthesis of rod-like nano-sized HA (nano-HA)

5.48 g of potassium phosphate dibasic trihydrate were dissolved in 100 mL of doubled distilled water. Successively, 0.2 vol.% of Darvan 821-A was added to the solution and the pH was set to 10.5 through the addition of 1 M sodium hydroxide solution. In order to have a Ca/P molar ratio of 1.67, a second solution obtained by dissolving 4.44 g of calcium chloride in 60 mL of doubled distilled water was prepared and, after 1 hour of stirring, added dropwise into the solution containing the phosphate precursor while constantly maintaining the pH at 10.5 through the addition of sodium hydroxide. The resulting solution was kept under stirring for 3 hours, constantly maintaining the pH at 10.5. After an overnight ageing step performed at atmospheric pressure and room temperature, the supernatant was removed, and the remaining slurry was poured in a 250 mL Teflon-lined hydrothermal reactor and placed in an oven at 100°C for 4 hours. After cooling, the supernatant was removed, and the resulting slurry was centrifuged in order to separate HA particles. The latter were washed three times with distilled water and once with pure ethanol. In the end, nano-HA particles were collected in a Petri dish and dried in an oven at 100°C for 24 hours [3].

2.2.2 Preparation of the type I collagen-based suspensions

All the collagen-based systems were developed using Type I collagen powders derived from bovine Achilles tendon (Blafar Ltd, Dublin, Ireland).

The amount of collagen powders and inorganic phase particles used to formulate the hybrid systems were calculated considering the volume percentages of the organic and inorganic phases in the natural bone tissue (53% vol. of collagen and 47% vol. of inorganic phase) [9].

All the suspensions were realised following the steps reported in Figure 2.2, obtaining a final concentration of collagen of about 1 wt% and 1.5 wt%. The inorganic phase dispersion step was optimised according to the physico-chemical properties of the particles and their tendency to aggregate in order to avoid large agglomerates. All the steps of the protocols were performed keeping the temperature under 10 °C and using pre-chilled reagents in order to avoid collagen denaturation and its premature gelation.

The specific amount of collagen and inorganic phases as well as the different strategies for the proper dispersion are following reported for each developed system.

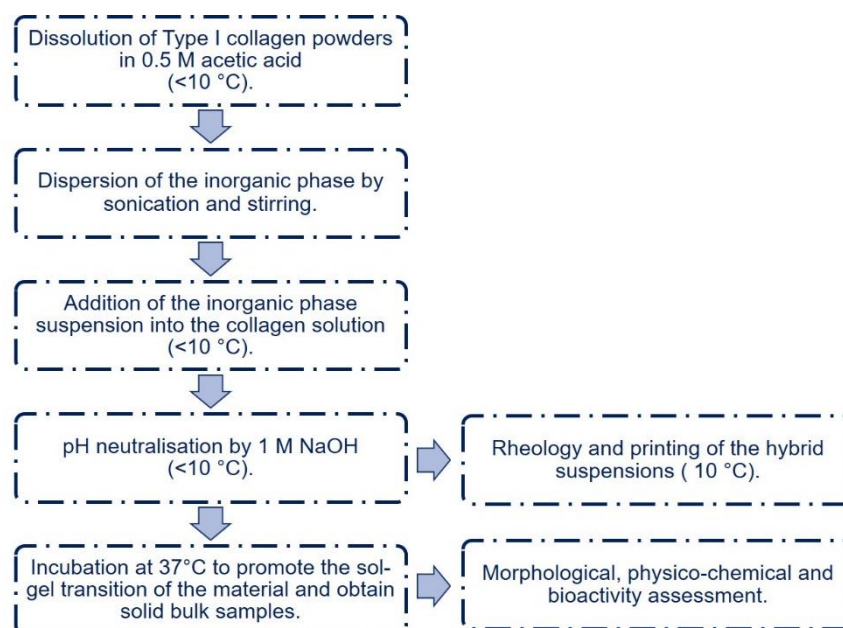


Figure 2. 2 General protocol used to prepare the collagen-based hybrid suspensions.

Preparation of collagen-based suspensions containing Sr-MBGs (Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%)

The final weight amounts of the different materials were calculated respecting the volume ratio of organic and inorganic phase in bone [1,2] and considering densities of about 1.4 g/cm³, 2 g/cm³ and 1 g/cm³ for collagen powders, MBG_SD_Sr4% and MBG_SG_Sr4%, respectively. According to this, the final formulations consisted in final concentrations of 61.8 wt% of type I collagen and 38.2 wt% Sr-MBG particles. According to manufacturer's instructions, type I collagen powders derived from bovine Achilles tendon (Blafar Ltd., Dublin, Ireland) were dissolved in 0.5 M acetic acid solution under stirring at about 4 °C overnight, until complete dissolution. MBG_SD_Sr4% or MBG_SG_Sr4% particles were dispersed in 0.5 M acetic acid prior to addition into the collagen solution. To avoid particle aggregation, MBG_SD_Sr4% suspension was stirred for 1 hour and sonicated for 30 minutes while MBG_SG_Sr4% suspension was stirred for two hours and sonicated for 1 hour using an ultrasonic bath (Digitec DT 103H, Bandelin). The resulting Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% suspensions were left stirring for about one hour in order to ensure optimal homogeneity, as illustrated in Figure 2.3.

To promote the sol-gel transition and the collagen fibre reconstitution, the acidic pH of the suspensions was subsequently neutralised by adding 1 M NaOH solution reaching a final collagen concentration of about 1 wt% and 1.5 wt%. The suspensions were stored at 4 °C until used.

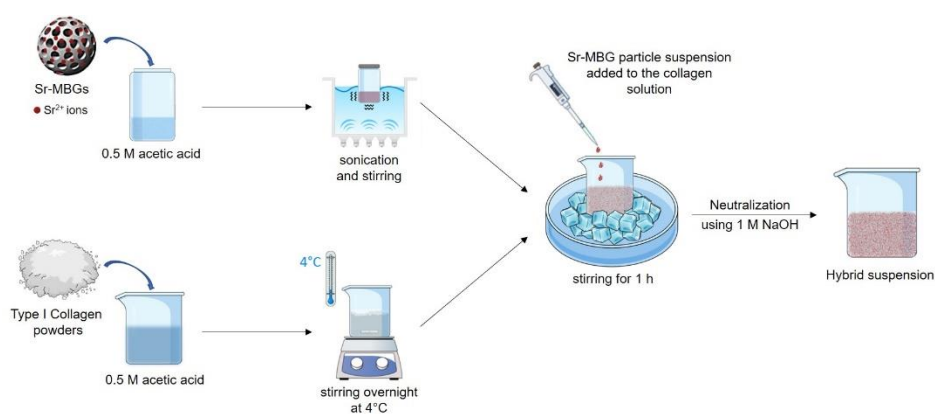


Figure 2. 3 Preparation of Sr-MBG containing collagen-based systems [4].

Preparation of collagen-based suspensions containing rod-like nano-HA (Coll/nano-HA)

The weight amounts of collagen powders and nano-HA particles were calculated considering densities of about 1.4 g/cm³ and 2.9 g/cm³, obtaining final concentrations of 35 wt% and 65 wt%, respectively [3]. In details, nano-HA particles were suspended in 1 M NaOH containing Darvan 821-A by sonicating for 1 hour using an ultrasonic bath (Digitec DT 103H, Bandelin, Berlin, Germany) and subsequently stirring overnight to promote the proper dispersion of nano-HA particles. Different concentrations (0 vol.%, 0.5 vol.% and 3 vol.%) of Darvan 821-A were tested in order to define the most appropriate in terms of particle dispersion and final composite properties. The resulting nano-HA suspension was subsequently combined with the type I collagen solution, previously obtained by dissolving collagen in 0.5 M acetic acid under stirring at 4°C overnight. To promote the physiological reconstitution of collagen fibres, the pH of the suspension was adjusted to 7.4 by adding the proper amount of 1 M NaOH solution and reaching a final collagen concentration of 1.5 wt%. The obtained suspension was stored at 4 °C until used.

Preparation of the collagen-based suspension containing MBG_SG_Sr4% and nano-HA (Coll/MBG_SG_Sr4%/nano-HA)

Starting from the volume ratio between organic and inorganic phases in the natural bone (53% vol. of collagen and 47% vol. of inorganic phase) [9,10] and considering a ratio of 50:50 between nano-HA and MBG_SG_Sr4% particles, 45 wt% of type I collagen powders, 40 wt% of nano-HA and 15 wt% of MBG_SG_Sr4% were calculated as final weight percentages.

To obtain the final suspension, collagen powders were firstly dissolved in 0.5% acetic acid at 4 °C overnight. Parallely, nano-HA particles were resuspended in a mixture of 1 M NaOH and Darvan 821-A (0.5% v/v of the final volume) by sonicating for 1 hour and stirring overnight to promote the proper dispersion of the inorganic particles. MBG_SG_Sr4% were resuspended in 0.5 M acetic acid, sonicated for 1 hour and stirred for 2 hours prior to mixing with the collagen solution. After the addition of the MBG_SG_Sr4% suspension, the mixture was

stirred for about 1 hour to ensure optimal homogeneity. Subsequently, the nano-HA suspension was added to the collagen/MBG_SG_Sr4% mixture dropwise, almost leading to the pH neutralisation. The pH of the suspension was further adjusted to 7.4 adding few drops of 1 M NaOH. The final suspension was stored at 4 °C until used.

2.2.3 Preparation of methacrylated collagen-based formulations for UV curing applications

With the aim to enhance the stability of the deposited structures, the 3D printing of UV curable systems was explored as alternative strategy.

To enable the UV curing of the materials, methacrylated collagen (COLMA) powders derived from bovine Achilles' tendon (PhotoCol-UV Methacrylated collagen, Advanced BioMatrix, USA) were used to prepare hybrid formulations respecting the weight ratio between collagen, MBGs and nano-HA previously calculated as reported in Section 2.2.2. The protocols were optimised following the instructions reported by the collagen provider (Advanced BioMatrix) to obtain the best dissolution and stability of the collagenous solution. Moreover, different concentrations (0.1%, 0.5%, 1%) of a cytocompatible photoinitiator (Irgacure 2959, Advanced BioMatrix, USA) were added to the material formulation to promote the crosslinking of collagen upon UV light application. The different hybrid formulations were prepared as follows:

1.5%COLMA

To prepare the methacrylated collagen solution, COLMA powders were dissolved in 20 mM acetic acid stirring at 4 °C overnight. 100 mg of IRGACURE 2959 were dissolved in 1 mL of neat methanol and the calculated volume of photoinitiator solution was mixed with a neutralisation buffer consisting of an alkaline 10X phosphate buffered saline (PBS) solution. The resulting mixture was added to the methacrylated collagen solution reaching a final pH in the range of 7-7.4. All the calculations were made in order to obtain a final concentration of 1.5 wt% of COLMA.

1.5%COLMA/MBG_SG_Sr4%

To prepare the hybrid formulation, COLMA powders were dissolved in 20 mM acetic acid stirring at 4 °C overnight and MBG_SG_Sr4% were dispersed in 20 mM acetic acid stirring for 2 hours and sonicating for 1 hour. The MBG_SG_Sr4% suspension was subsequently added to the COLMA solution and kept stirring to obtain a homogeneous suspension. 100 mg of IRGACURE 2959 were dissolved in 1 mL of neat methanol and the calculated volume of photoinitiator solution was mixed with the neutralisation buffer (10X PBS). The resulting mixture was added to the COLMA/MBG_SG_Sr4% suspension reaching a final pH in the range of 7-7.4. All the calculations were made in order to obtain a final concentration of 1.5 wt% of COLMA and respecting the ratio between the inorganic and organic phase (53% vol. of collagen and 47% vol. of inorganic phase) used in Section 2.2.2 [9,10].

1.5%COLMA/nano-HA

To prepare the hybrid formulation, COLMA powders were dissolved in 20 mM acetic acid stirring overnight. 100 mg of IRGACURE 2959 were dissolved in 1 mL of neat methanol and the calculated volume of photoinitiator solution was mixed with the neutralisation buffer (10X PBS) and Darvan 821-A (final concentration of 0.5% v/v). The nano-HA powders were then dispersed in the resulting solution sonicating for 1 hour and stirring overnight to avoid particle aggregation. The nano-HA suspension was added to the previously obtained COLMA solution reaching a final pH in the range of 7-7.4. All the calculations were made in order to obtain a final concentration of 1.5 wt% of COLMA and respecting the ratio between the inorganic and organic phase used in Section 2.2.2.

2.2.4 Preparation of bulk samples for material characterisation

Bulk samples of the developed materials were prepared to investigate the morphological, physico-chemical, bioactivity and rheological properties of the composite materials after the reconstitution of a solid gel at 37 °C. To prepare the samples, 400 µL of the specific suspension were pipetted in a silicon mould, subsequently incubated at 37 °C for 3 hours in order to allow the sol-gel transition of the system resulting from the natural physical crosslinking of collagen molecules [11,12]. The solid samples were then collected and stored at 4 °C until tested.

In details, circular samples measuring 10 mm in diameter and 5 mm in thickness were used to perform the following analyses:

- Morphological characterisation before and after the chemical crosslinking.
- Physico-chemical characterisation before and after the chemical crosslinking.
- Bioactivity tests on Sr-MBG containing systems.
- Strontium ion release tests before and after the chemical crosslinking treatment.
- Hydrolytic degradation tests before and after the chemical crosslinking.
- Enzymatic degradation tests before and after the chemical crosslinking.

On the contrary, samples measuring 20 mm in diameter and about 1 mm in thickness were used to perform the rheological tests in order to fit the geometry of the rotational rheometer, as described in Section 2.2.11. In this case, 600 µL of the specific suspension were pipetted in a silicon mould and subsequently incubated at 37 °C for 3 hours.

2.2.5 Strategies for the chemical crosslinking of collagen

Chemical crosslinking by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) and N-hydroxysuccinimide (NHS)

The chemical crosslinking of collagen by means of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was investigated following procedures previously

reported in the literature [13,14]. In detail, bulk samples of Coll/MBG_SD_Sr4%, produced exploiting the natural self-assembly of collagen as reported in Section 2.2.4, were incubated in 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) (Sigma Aldrich, Italy) and 25 mM N-hydroxysuccinimide (NHS) (Sigma Aldrich, Italy) in 98% ethanol at 37 °C for 24 hours. The chemically treated samples were subsequently washed two times with distilled water to remove any residuals of the crosslinking agent and stored at 4 °C until performing further analyses.

Chemical crosslinking by 4-star poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG)

The chemical crosslinking of collagen with 4-star poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG) (Abbexa Ltd., Cambridge, UK) was explored and optimised based on procedures previously reported in the literature [15]. In details, bulk samples of Coll/MBG_SG_Sr4% produced as described in Section 2.2.4 were immersed in 3 mL of Dulbecco phosphate buffered saline (D-PBS, Sigma Aldrich) containing 1 wt% of 4-StarPEG at 37 °C for 24 h. After the crosslinking treatment, the samples were washed two times in distilled water under mild agitation to remove any residuals of the crosslinking agent and stored at 4 °C until performing further analyses.

Chemical crosslinking by genipin

Two different solutions were prepared by dissolving genipin powders (Challenge Bioproducts, Taiwan) in Dulbecco Phosphate Buffered Saline (D-PBS, Sigma Aldrich) (GEN/PBS) or 70% ethanol in water (GEN/EtOH). A genipin concentration of 0.5 wt% and the crosslinking procedure were optimised based on previous studies reported in the literature [15–17]. Genipin was used to promote collagen crosslinking and the two different solvents were compared to investigate their effect on strontium ion release that should be hindered during the crosslinking process in the case of Sr-MBG containing systems.

Bulk samples of Coll/MBG_SG_Sr4% were chemically crosslinked by immersion in 0.5 wt.% GEN/PBS or 0.5 wt.% GEN/EtOH solutions at 37 °C for 24 h. The samples were then washed two times in distilled water and under mild agitation, to remove genipin residues.

In detail, Coll/MBG_SG_Sr4% samples were tested using both 0.5 wt.% GEN/PBS and 0.5 wt.% GEN/EtOH crosslinker solutions to investigate the potential influence of the medium over the strontium ion release kinetics.

Based on the higher crosslinking efficiency obtained with the Coll/MBG_SG_Sr4% system, the chemical crosslinking with genipin of Coll/nanoHA and Coll/MBG_SG_Sr4%/nanoHA systems was obtained using only 0.5 wt.% GEN/EtOH, after optimisation of the crosslinking strategy.

Similarly, the use of 0.5% GEN/EtOH was selected for the chemical crosslinking of all the 3D printed scaffolds, as reported in Section 2.2.12.

2.2.6 Morphological assessment by means of Field Emission Scanning Electron Microscopy (FESEM)

In this study, Field Emission Scanning Electron Microscopy (FESEM) was used to investigate the reconstitution of the collagen fibrils after the sol-gel transition and the chemical crosslinking of the developed systems as well as the dispersion of the inorganic particles into the collagenous matrix.

This technique enables the non-destructive analysis of the morphological features of the samples up to the nanoscale at very high resolutions. To improve the final images, samples are normally coated with very thin layers of conductive materials (such as gold, chromium, and platinum) in order to reduce the potential surface charging especially in the case of non-conductive specimens.

For the analysis, all the samples were frozen at -20°C and lyophilized for 24 h using a Lyovapor L-200 freeze-dryer (Büchi, Switzerland) under vacuum (< 0.1 mbar). The lyophilized samples were then coated with a 7 nm thin platinum layer and analysed by Field-Emission Scanning Electron Microscopy (FESEM) using a ZEISS MERLIN instrument.

In details, FESEM was used to observe the reconstitution of collagen fibrils and the dispersion of the inorganic phase for all the developed systems and scaffolds, before and after the chemical crosslinking treatments. In addition, FESEM was performed to detect the potential deposition of HA crystals in Sr-MBG containing samples collected after bioactivity tests in Simulated Body Fluid (SBF) as following described in Section 2.2.8.

2.2.7 Physico-chemical and structural analyses: attenuated total reflection ATR-FTIR and X-Ray Diffraction (XRD)

ATR-FTIR analysis

Attenuated total reflection (ATR) combined with infrared spectroscopy enables the direct chemical identification of samples in the solid or liquid state. FTIR analysis in the attenuated total reflection mode (ATR) was performed to detect the potential formation of chemical interactions between the different components of the systems as well as after the crosslinking treatments.

FTIR spectra, in the $4000\text{--}650\text{ cm}^{-1}$ range, were collected by using a Bruker Equinox 55 spectrometer, equipped with MCT cryodetector, at a spectral resolution of 4 cm^{-1} and accumulation of 32 scans, by using the attenuated total reflection (ATR) mode.

For the analysis, samples were frozen at -20°C and lyophilized for 24 h using a Lyovapor L-200 freeze-dryer (Büchi, Switzerland) under vacuum (< 0.1 mbar).

In details, ATR-FTIR was used to perform the chemical characterisation of all the different composite materials before and after chemical crosslinking treatments and to detect the potential deposition of calcium phosphates in Sr-MBG containing samples after bioactivity tests.

X-Ray Diffraction measurements (XRD)

The X-ray diffraction (XRD) analysis is exploited to obtain qualitative and quantitative information about the structure of the sample, defining the presence of broader or sharper peaks associated with the presence of amorphous or crystalline phases, respectively.

Wide-angle X-Ray Diffraction measurements (XRD) were performed employing a CuK α radiation emitted at 40 kV and 40 mA. The investigated angular range was set from 10 to 80 2 θ degrees, with a step size of 0.013 2 θ degrees and a scan step time of 45 s. XRD spectra analysis for phase identification was performed through the X'Pert HighScore software.

For the analysis, samples were frozen at – 20°C and lyophilized for 24 h using a Lyovapor L-200 freeze-dryer (Büchi, Switzerland) under vacuum (< 0.1 mbar).

In details, XRD was exploited to confirm the potential deposition of HA nanocrystals in Sr-MBG containing systems after bioactivity tests. The graphs obtained were compared with the one of HA as a reference using the peaks identified by the International Centre for Diffraction Data [18,19].

2.2.8 Bioactivity assessment

The bioactivity of the hybrid formulations containing Sr-MBGs was investigated by soaking bulk samples prepared as reported in Section 2.2.4 in Simulated Body Fluid (SBF) and observing the potential deposition of HA nanocrystals at different time points by means of FESEM, ATR-FTIR and XRD analyses.

The bioactivity assessment was performed according to previous studies investigating the bioactive properties of collagen-based systems reported in the literature and previous tests conducted on Sr-MBG particles alone [9,10,14,37].

In details, bulk samples prepared as described in Section 2.2.4 were incubated in 3 mL of SBF at 37 °C and at 50 rpm for up to seven days. At each time point (3 h, 1 day, 3 days, and 7 days) the samples were rinsed in distilled water twice to remove salt residuals and collected for further analyses. To allow the proper comparison between the bioactive features of Sr-MBGs alone and Sr-MBGs embedded in the collagenous matrices, each sample was immersed in 3 mL of SBF obtaining a final particle concentration of about 1 mg/mL. The experiment was carried out in triplicate. To evaluate the HA crystal formation by FESEM, ATR-FTIR, and XRD analyses, the collected samples were abundantly washed in distilled water, frozen at – 20 °C and subsequently lyophilised for 24 h as reported in the previous sections.

The bioactivity assessment was carried out on the bulk samples of Coll/MBG_SD_Sr4%, Coll/MBG_SG_Sr4% and Coll/MBG_SG_Sr4%/nano-HA prepared as reported in Section 2.2.4.

2.2.9 *In vitro* degradation tests

The stability of the physically and chemically crosslinked systems was tested by using hydrolytic and enzymatic degradation tests. The percentage of weight loss was measured after incubation of bulk samples prepared as reported in Section 2.2.4 in D-PBS and collagenase solutions at different time points.

The samples collected at each time point were lyophilized and accurately weighted to record the weight after enzymatic digestion and hydrolytic degradation. After weighting lyophilized non-treated samples, weight loss was assessed by using the following formula:

$$W = [(W_0 - W_d)/W_0] * 100$$

where W_0 represents the initial mass of the sample, W_d represents the mass after degradation and W is the resultant loss percentage. Three samples were considered for each time point, and results were reported as mean $\pm$ standard deviation.

Enzymatic degradation

Collagenase from *Clostridium histolyticum* (type I, Sigma Aldrich) was used for the enzymatic degradation study of the different systems, immersing each sample in 1 mL of DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich) containing 1 mg of collagenase (2.1 units) and subsequently incubated at 37 °C under mild agitation (50 rpm) [20,21]. At predefined time points (12, 24 and 48 h) samples were collected and abundantly washed in distilled water and then frozen.

The enzymatic degradation was assessed on Coll/MBG_SG_Sr4% samples before and after the chemical crosslinking with 4-StarPEG, GEN/PBS and GEN/EtOH, as well as on non-crosslinked and GEN/EtOH crosslinked Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA samples.

Hydrolytic degradation

D-PBS was selected as the medium for performing hydrolytic degradation tests, by immersing the samples in 3 mL of D-PBS, at 37 °C, under mild agitation (50 rpm) [20,21]. The samples were collected at predefined time points (24 h, 72 h, 7 days and 14 days) and frozen.

The hydrolytic degradation was assessed on Coll/MBG_SG_Sr4% samples before and after the chemical crosslinking with 4-StarPEG, GEN/PBS and GEN/EtOH, as well as on non-crosslinked and GEN/EtOH crosslinked Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA samples.

2.2.10 Strontium ion release tests

The kinetics of strontium ion release from the Sr-MBG containing systems before and after chemical crosslinking were monitored and compared to those observed for the Sr-MBG matrices alone. For the analysis, Tris HCl buffer (Tris(hydroxymethyl)aminomethane (Trizma) (Sigma Aldrich, Milan, Italy) 0.1 M, pH 7.4) was selected as medium according to the method previously optimised by

the IRIS group to assess the release from the particles alone. Based on the previous experiments, each sample was immersed in 3 mL of the release medium considering a final concentration of 1 mg/mL of particles, keeping a constant temperature of 37 °C and a mild agitation of 50 rpm.

At predefined time steps (10 hours, 24 hours, 3 days, 7 days) the medium was collected and entirely refreshed. To measure the concentration of released Sr ions, the collected supernatant was analysed by the inductively coupled plasma atomic emission spectrometry technique (ICP-AES) (ICP-MS, Thermoscientific, ICAP Q, Waltham, MA, U.S.A) after proper dilutions.

For the chemically treated systems, samples were incubated up to 24 hours in 3 mL of the crosslinking solution in order to investigate the potential strontium release already during the chemical crosslinking step. To this aim, the release of strontium ions was firstly measured during the chemical treatment with 4-StarPEG, GEN/PBS and GEN/EtOH solutions carried out up to 24 hours and subsequently by soaking the crosslinked samples in Tris HCl buffer.

The percentage of ion released was calculated by considering the amount of strontium initially incorporated in MBG particles and equivalent to 4% molar percentage, as well as the content of inorganic phase incorporated into the hybrid formulation. To confirm the theoretical data, analogous samples were dissolved in a mixture of nitric and hydrofluoric acids and subsequently analysed, via ICP, in order to calculate the final percentage of the released ions starting to the ppm values measured during the analysis.

Analyses were conducted in triplicate, and the percentages of released ions are reported as mean value $\pm$ standard deviation. The resulting data were compared with the release patterns previously obtained from the Sr-MBG matrices alone.

The strontium release tests were performed on bulk samples of all the developed Sr-MBG containing systems, where the extent of ion loss in the different chemical crosslinking baths was explored only using Coll/MBG_SG_Sr4%.

2.2.11 Rheology of the collagen-based systems

Rheology studies the deformation and flow of matter subjected to different environmental and stress conditions. Rheological tests can be performed on liquid and solid materials studying the variation of their visco-elastic properties at different shear stresses, shear rates and temperatures applied by means of a rotational rheometer [22].

In details, flow tests are normally performed on fluids to investigate the variation of the material viscosity modulating the rotational motion of the geometry at the desired temperature. The resulting viscosity η of the material is measured considering the constitutive equation reported in Figure 2.4 and is dependent on the following factors:

- applied torque M (Nm)
- geometric constant K_G ($2/\pi r^3$), where r is the radius of the geometry
- angular velocity of the motor Ω (rad/s)

- geometric constant K_γ (r/h), where r is the radius of the geometry and h the gap between the geometry and the sample loading plate

$$\eta = \frac{\sigma}{\dot{\gamma}} = \frac{M \cdot K_\sigma}{\Omega \cdot K_\gamma}$$

Rheological
Parameter
Constitutive
Equation
Raw rheometer
Specifications
Geometric
Shape
Constants

Figure 2. 4 Equation of the viscosity expressed by the rheometer specifications and the geometric shape constants.

On the contrary, oscillatory tests are performed on both fluids and solid samples to monitor the variation of the visco-elastic properties of the material at different stress conditions and predefined temperatures. In this case, the geometry applies an oscillatory stress dependent on the selected amplitude and frequency to measure the complex modulus of the material (G , Pa) described by its storage (G' , Pa) and loss (G'' , Pa) components. The resulting visco-elastic modulus G of the material is measured considering the constitutive equation reported in Figure 2.5 and is dependent on the following factors:

- applied torque M (Nm)
- geometric constant K_σ ($2/\pi r^3$), where r is the radius of the geometry
- angular deformation of the motor θ (rad)
- geometric constant K_γ (r/h), where r is the radius of the geometry and h the gap between the geometry and the sample loading plate

$$G = \frac{\sigma}{\gamma} = \frac{M \cdot K_\sigma}{\theta \cdot K_\gamma}$$

Rheological
Parameter
Constitutive
Equation
Raw rheometer
Specifications
Geometric
Shape
Constants

Figure 2. 5 Equation of the visco-elastic modulus expressed by the rheometer specifications and the geometric shape constants.

All the rheological tests were performed using a DHR-2 controlled stress rotational Rheometer (TA Instruments, Waters) equipped with a parallel plate geometry with a diameter of 20 mm and a Peltier plate system to constantly control the system temperature (Figure 2.6).

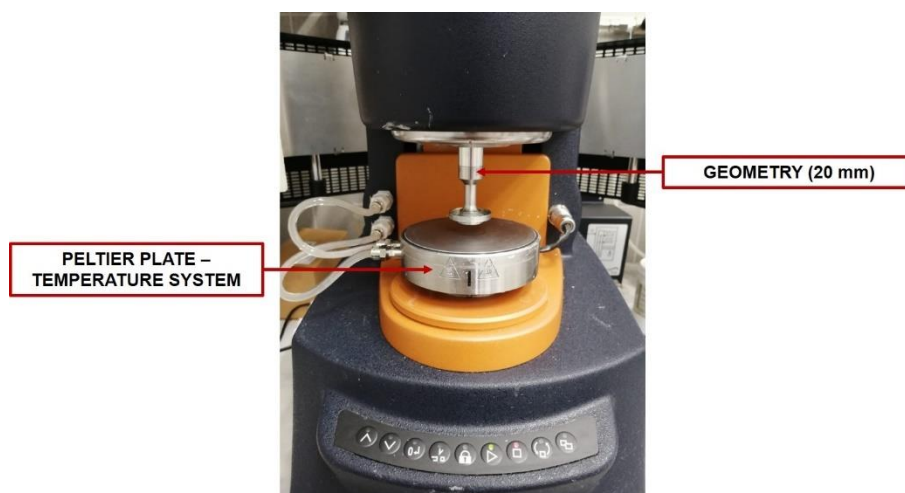


Figure 2. 6 DHR-2 Rotational Rheometer used for the rheological assessment.

Viscosity of the suspensions

The variation of the suspension viscosity was studied by means of flow tests performed varying the shear rates applied to the material samples at 10 °C [23]. Each test was performed in triplicate, setting a gap ranging between 400 μm and 1500 μm according to the volume of the sample loaded.

The suspension viscosity and yield stress were measured at different shear conditions performing *flow ramp tests* at 10 °C where shear rates values varied between 0.01 and 1000 s^{-1} .

Peak hold tests were performed at 10 °C to simulate the printing process and investigate the shear recovery of the suspensions by setting a shear stress value of 100 s^{-1} (100 s) and 0.1 s^{-1} (300 s) to reproduce the extrusion and the deposition step, respectively.

The system tested for each experiment has been summarised in Table 2.1.

Table 2. 1 Summary of the flow tests performed on the hybrid formulations.

RHEOLOGICAL TEST	SYSTEM TESTED (suspensions)	RHEOLOGICAL PROPERTY INVESTIGATED
Flow ramp (10 °C)	Coll/MBG_SD_Sr4%; Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA; 1.5%COLMA; 1.5%COLMA/MBG_SG_Sr4%; 1.5%COLMA/nano-HA	Viscosity; Yield point
Peak hold test (10 °C)	Coll/MBG_SD_Sr4%; Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA	Shear recovery

Visco-elastic properties of the hybrid formulations before and after chemical treatments

The visco-elastic properties of the different systems before and after crosslinking treatments were studied by means of oscillatory tests, monitoring the value and variation of the storage (G') and loss (G'') moduli at different stress and temperature conditions [23]. The tests were performed in triplicate, setting a gap ranging between 400 μm and 1500 μm according to the specific volume or thickness of the sample loaded.

Dynamic *amplitude sweep tests* performed on the suspensions kept at 10 °C were exploited as alternative and additional method to define the yield point. The tests were performed varying the oscillation stress between 0.1 and 100 Pa at a constant frequency of 1 Hz.

The sol-gel transition of the hybrid formulations at 37 °C was investigated by means of *time sweep tests* keeping a constant strain of 1% and frequency of 1 Hz. The suspensions were loaded on the plate at 10 °C to avoid premature system gelation while the test was performed at 37 °C for 1 hour.

Dynamic *amplitude sweep tests* (0.01–1% strain, 1 Hz) were conducted to investigate the increasing of storage (G') and loss (G'') moduli with increasing time of incubation at 37 °C (3 hours) and after the crosslinking treatments. The amplitude sweep test also allowed the detection of the linear visco-elastic region (LVR). For the analysis the samples, measuring 20 mm in diameter, were previously prepared as described in Section 2.2.4 and loaded on the Peltier plate.

Oscillatory *temperature ramps* ranging between 25 °C and 80 °C were performed under 1% strain and 1 Hz using a ramp rate of 5 °C/min to detect the denaturation temperature of the material associated to the sharp decrease of the overall visco-elastic modulus (G' , G''). For the analyses, samples measuring 20 mm in diameter, were previously prepared as described in Section 2.2.4 and loaded on the Peltier plate.

UV curing kinetics of the photocurable systems

For the UV curing tests, an external UV source, equipped with a 365 nm filter, was connected to the rheometer accessories (Figure 2.7) via an optical fibre cable. Exposure time and light intensity were set directly by the Trios Software after proper calibration of the UV source. The UV curing kinetics of the systems were obtained by means of oscillatory tests (1% strain, 1 Hz) at room temperature (RT). The suspensions were firstly loaded onto the support plate (quartz crystal), and then subjected to UV light at 365 nm according to the photoinitiator used, at 15 mW/cm² for 1200 seconds. UV curing tests were performed in triplicate and setting a gap ranging between 400 μm and 1500 μm according to the volume of the sample loaded.



Figure 2. 7 UV curing system used for the analysis.

Visco-elastic properties of the gelatine slurries used for the printing tests

The visco-elastic properties of the gelatine-based supporting materials (gelatine slurry FRESH V1; gelatine slurry FRESH V2) used for the printing process were analysed by means of oscillatory tests. For the analysis, a small volume of the slurry was loaded onto the Peltier plate and the gap was set accordingly, ranging between 600 μm and 1600 μm .

Oscillatory *frequency sweep tests* were performed in the range of 0.1 and 100 Hz at 1% strain and at 20 °C, to study the stability of the supporting materials.

The gel to sol transition was analysed by means of *temperature ramps* between 10 °C and 40 °C under 1% strain and 1 Hz using a ramp rate of 5 °C/min.

The systems tested for each experiment have been summarised in Table 2.2.

Table 2. 2 Summary of the oscillation tests performed on the different systems.

RHEOLOGICAL TEST	SYSTEM TESTED	RHEOLOGICAL PROPERTY INVESTIGATED
Time sweep test (37 °C)	Coll/MBG_SD_Sr4%; Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA; 1.5%COLMA; 1.5%COLMA/nano-HA; 1.5%COLMA/MBG_SG_Sr4%	Sol to gel transition (G', G'')
Amplitude sweep test (10 °C)	Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA	Yield point (G', G'')
Amplitude sweep test (37 °C)	Coll/MBG_SD_Sr4%; Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA; 4-StarPEG crosslinked Coll/MBG_SG_Sr4%; GEN/PBS crosslinked Coll/MBG_SG_Sr4%; GEN/EtOH crosslinked Coll/MBG_SG_Sr4%; GEN/EtOH crosslinked Coll/nano-HA; GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	Material strength (G', G''); LVR
Temperature ramp (25 °C – 80 °C)	Coll/MBG_SD_Sr4%; Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA; 4-StarPEG crosslinked Coll/MBG_SG_Sr4%; GEN/PBS crosslinked Coll/MBG_SG_Sr4%; GEN/EtOH crosslinked Coll/MBG_SG_Sr4%; GEN/EtOH crosslinked Coll/nano-HA; GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	Denaturation temperature (G', G'')
Temperature ramp (10 °C – 40 °C)	Gelatine slurry (version 1); Gelatine slurry (version 2)	Gel to sol transition (G', G'')
Frequency sweep test (20 °C)	Gelatine slurry (version 1); Gelatine slurry (version 2)	Gel stability (G', G'')
Time sweep test (RT) upon application of UV light (365 nm)	1.5%COLMA; 1.5%COLMA/MBG_SG_Sr4%; 1.5%COLMA/nano-HA	UV curing kinetics (G', G'')

2.2.12 Printing tests using extrusion-based systems

3D printing techniques are classified as additive manufacturing technologies, able to realise 3D constructs starting from computer-aided design (CAD) models and by deposition or assembling of the constitutive materials layer by layer.

Specifically, for extrusion-based systems, the materials are dispensed in continuous filaments either pneumatic, piston- or screw-driven [24]. The final shape fidelity and resolution of the printed structure will largely be influenced by the selection of the proper nozzle diameter and printing parameters.

2.2.12.1 Printing tests exploiting the freeform reversible embedding of suspended hydrogel method (FRESH V1, FRESH V2)

The printing process was developed and optimised based on a method previously described in the literature and reported as freeform reversible embedding of suspended hydrogels (FRESH) [25,26]. Based on this, the printability of the developed collagen-based suspensions was assessed using a commercial BIOX 3D Bioprinter (Cellink, Gothenburg, Sweden) and extruding the suspensions into gelatine-based supporting baths kept at room temperature as schematically represented in Figure 2.8. Two different gelatine slurries (gelatine slurry FRESH V1; gelatine slurry FRESH V2) were tested for the printing process. After printing, the scaffolds were obtained by incubating at 37 °C promoting the sol-gel transition of the hybrid formulations and the removal of the supporting bath. After subsequent washing steps in distilled water, the designed 3D printed scaffolds were ready for further processing.

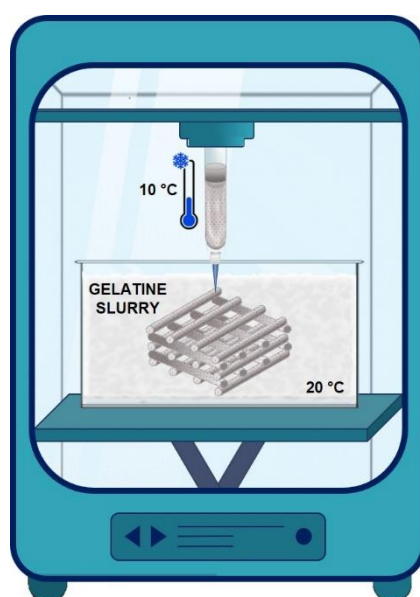


Figure 2. 8 Schematic representation of the 3D printing process exploiting the FRESH method.

Preparation and characterisation of the gelatine slurry FRESH V1

The protocol to prepare the gelatine slurry was optimised following the instructions reported in the patent and subsequent publications by Feinberg and co-workers [25,26]. To prepare the slurry, a 4.5 wt% gelatine solution was initially obtained by dissolving porcine gelatine powders (Type A, Sigma Aldrich) in 300 mL of Dulbecco Phosphate Buffered Saline (D-PBS, Sigma Aldrich) pre-heated at 37 °C. After complete dissolution, the gelatine solution was kept at 4 °C overnight to obtain a solid gel. Then, 200 mL of prechilled D-PBS was added to the gelled gelatine in a 500 mL jar, and everything was blended for 120 s. The resulting gelatine suspension was transferred into 50 mL conical tubes and centrifuged (3800

rcf, 4 min) at 4 °C, to collect the slurry particles, after accurately removing the supernatant. The gelatine particles were subsequently resuspended in precooled D-PBS and centrifuged again at 3800 rcf. The last two steps were repeated until completely removal of the foam present was obtained. The finally collected gelatine slurry was then stored at 4 °C until use. Immediately before use, the slurry was centrifuged at 225 rcf for 5 minutes and poured into 2-wells chamber slides used for the printing tests (Figure 2.9).

The volume of slurry poured in the container was regulated according to the 3D model dimensions, always obtaining the supporting bath at least 2 mm higher than the scaffold thickness.

Rheological tests were performed on the resulting gelatine slurry to investigate the peculiar visco-elastic properties as described in Section 2.2.11.

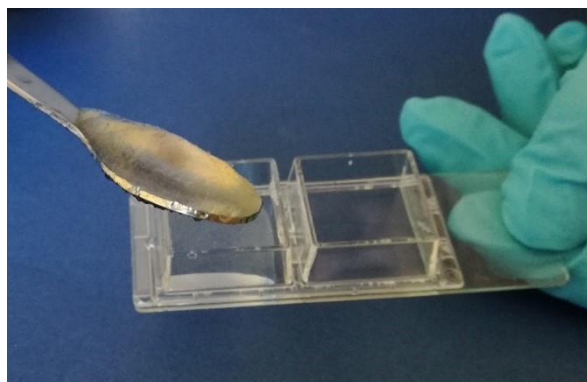


Figure 2. 9 The resulting gelatine slurry (FRESH V1) poured into 2-wells chamber slides before printing.

Preparation and characterisation of the gelatine slurry FRESH V2

The gelatine slurry FRESH V2 was obtained starting from the dried LifeSupport powders (FluidForm, Acton MA, USA) composed of gelatine microparticles of defined size and shape, and following the provider instructions for preparation [27].

Firstly, 1 g of LifeSupport dried powders were rehydrated in 35 mL of prechilled D-PBS for 10 minutes using 50 mL falcon tubes. The suspension was centrifuged at 600 rcf for 5 minutes to compact the particles at the bottom of the tube enabling the removal of the supernatant. The tube was shaken for few seconds until the hydrated particles started flowing and subsequently centrifuged at 1000 rcf for 5 minutes. After aspiration of the residual supernatant, the resulting gelatine slurry was poured into the desired container (2-wells chamber slides) avoiding the introduction of air bubbles.

The volume of slurry poured in the container was regulated according to the 3D model dimensions, always obtaining the supporting bath at least 2 mm higher than the desired scaffold thickness.

Rheological tests were performed on the resulting gelatine slurry to investigate their visco-elastic properties as described in Section 2.2.11.

Preparation of the collagenous suspensions for the printing tests

The suspensions prepared as described in Section 2.2.2 were stored at 4 °C until used. To decrease the amount of air bubbles present in the material, different strategies have been investigated:

- The suspensions were loaded into 6 mL syringes and kept under mild vacuum (using a vacuum chamber) for 24 hours at 4 °C.
- The suspensions were loaded into 6 mL syringes and subsequently centrifuged at 3000 rpm for 5 minutes keeping a constant temperature of 4 °C.
- The suspensions were stirred for 1 hour at 4 °C, loaded into 6 mL syringes and centrifuged at 3000 rpm at 4 °C for 5 minutes.

The suspensions were subsequently transferred into 3 mL printing cartridges and further centrifuged at 3000 rpm for 5 minutes before starting the printing process.

Printing tests

All the printing tests were performed using an extrusion-based system equipped with a temperature controlled pneumatic printhead mounted on a BIOX 3D Bioprinter as shown in Figure 2.10. Metal needles of 27 G and 30 G, having an inner diameter of about 200 µm and 120 µm respectively and measuring 12.22 mm in length, were used for the tests to enhance the final structure resolution.

The print-head temperature was set at 10 °C while the print-bed was maintained at 20 °C in order to keep constant the viscoelastic properties of the biomaterial ink and the gelatine-bath during the entire process.

The suspensions were loaded into 3 mL cartridges as previously described and mounted into the bioprinter after tightening the selected needle. All the cartridges and needles used for the tests were provided by the bioprinter manufacturers.

3D digital models for square scaffolds (10 x 10 mm surface) and up to 5 mm in thickness were selected from the .STL files library provided by the bioprinter manufacturer. A grid pattern and different percentages of infill (15%, 25%) were configured to obtain 3D structures showing pores of 2.2 mm and 1.5 mm, respectively.

The optimisation of the extrusion parameters was obtained varying print-head speed (7 - 15 mm/s), air pressure (20 - 100 kPa) and layer height (100 – 200 µm) according to the specific biomaterial ink and the resulting accuracy of the filament extruded. Both the printing settings and the 3D model features were configured using the integrated software (heartos_v1.7.0+165-dev) of the bioprinter.

Immediately before printing, a manual calibration was carried out positioning the needle at the centre of the container and setting a distance between the needle and the well bottom equal to the layer height. An empty container was used to facilitate the calibration operations that was immediately substituted by the chamber slide containing the gelatine slurry before printing.

Table 2.3 reports the formulations processed with the two different FRESH methods for the printing tests.

Table 2. 3 Hybrid formulations tested with the different FRESH methods.

PRINTING METHOD	SYSTEM TESTED
FRESH V1 (gelatine slurry version 1)	Coll/MBG_SG_Sr4%; Coll/nano-HA; Coll/MBG_SG_Sr4%/nano-HA
FRESH V2 (gelatine slurry version 2)	Coll/MBG_SG_Sr4%/nano-HA

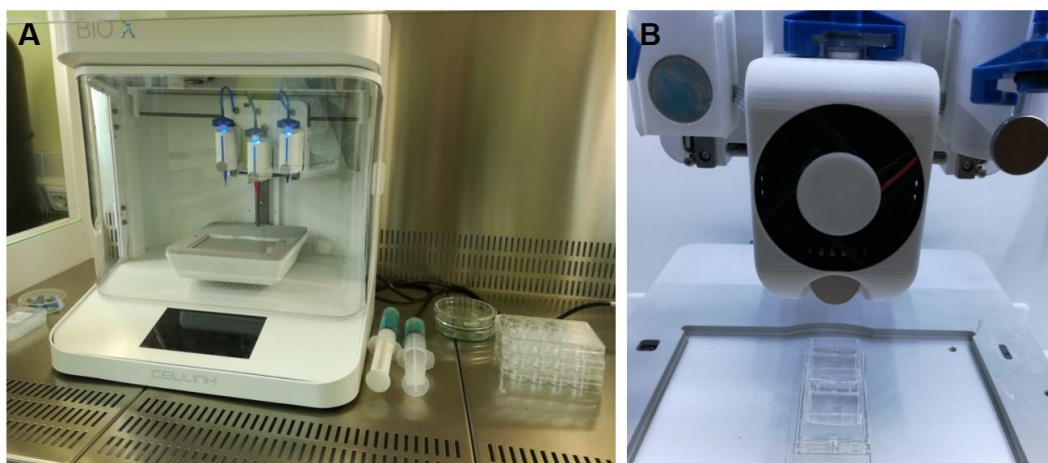


Figure 2. 10 The BIOX 3D Bioprinter (A) and the temperature controlled pneumatic printhead (B) used for the printing tests.

Scaffold design and post processing

After printing, the container (2-wells chamber slide) was removed from the bioprinter and incubated for 3 hours at 37 °C in order to promote the sol-gel transition of the collagenous systems while melting and subsequently removing the gelatine slurry.

To remove any residuals of gelatine, the 3D printed structures were washed two times in distilled water under mild agitation (50 rpm) for 30 minutes.

The scaffolds were visually inspected and imaged both before and after the removal of the supporting material in order to assess the shape fidelity on the x, y and z axes.

To increase the mechanical and thermal stability, the 3D constructs were chemically crosslinked by means of the 0.5% GEN/EtOH solution following the procedure optimised and specifically described in Section 2.2.5. Chemically crosslinked scaffolds were subsequently frozen at – 20 °C and lyophilised for 24 hours to enable the morphological assessment through FESEM as described in Section 2.2.6.

Preparation of scaffolds for the preliminary biological assessment

To perform a preliminary biological assessment, 3D printed scaffolds were realised extruding Coll/MBG_SG_Sr4%/nano-HA suspension at 30 kPa and 11 mm/s into the gelatine slurry FRESH V1, to obtain final constructs presenting 10 x 10 mm surface, 1 mm thickness and 30% internal infill density (about 1 mm pore diameter). After printing, the scaffolds were incubated at 37 °C for 3 hours, the gelatine was removed and two subsequent washing steps with distilled water were carried out to eliminate any supporting bath residuals. The scaffolds were finally

crosslinked by incubation in 3 mL of 0.5% GEN/EtOH at 37°C for 24 hours. The crosslinking bath was then removed, and the resulting constructs were washed two times in PBS and stored at 4°C until performing the tests.

For the scaffold preparation, the BIOX Bioprinter was placed under a laminar flow biological hood and sterile conditions were guaranteed during the entire manufacturing process.

2.2.12.2 Preliminary assessment of UV curable hybrid formulations for 3D extrusion printing

An alternative printing strategy based on the use of UV curable systems was preliminary assessed to potentially increase the stability of the designed scaffolds while printing. To this aim, hybrid formulations based on methacrylated collagen were developed and characterised before the optimisation of the printing process.

Before printing, COLMA suspensions containing 0.5% Irgacure 2959 and prepared as reported in Section 2.2.3 were loaded in 3 mL UV-shielding cartridges, centrifuged at 3000 rpm for 5 minutes to remove air bubbles, and mounted on the bioprinter.

For the printing tests, a temperature controlled pneumatic printhead kept at 10 °C was used to extrude the suspensions while a BIOX Photocuring Toolhead at a specific wavelength of 365 nm was exploited to cure the printed layers.

The gelatine slurry (FRESH V2) was poured into 2-wells chamber slides and the print-bed temperature was kept at 20 °C for the entire process.

3D digital models for square scaffolds (10 x 10 mm surface) and 1 mm thick were selected from the .STL files library provided by the bioprinter manufacturer. A 15% grid infill was configured to obtain 3D structures showing pores of about 2.2 mm. The printing tests were performed with 27 G needles and the parameters (air pressure, printhead speed, layer height) optimised by visual observation of the printing resolution of each deposited layer.

To maximise the effect of UV irradiation, the UV curing was set to be active layer by layer, with the UV source centred at the deposited layer. The UV power was adjusted to 100% (about 15 mW/cm²) selecting an exposure time of 120 seconds and distance from the layer of 1 cm. In summary, the UV curing parameters were selected in order to have the maximum power, the shortest distance and the longest exposure time supported by the bioprinter.

Both the printing and UV curing settings were configured using the integrated software (heartos_v1.7.0+165-dev) of the bioprinter.

Immediately before printing, a manual calibration was carried out positioning the needle at the centre of the container and setting a distance between the needle and the well bottom equal to the layer height. An empty container was used to facilitate the calibration operations that was immediately substituted by the chamber slides containing the gelatine slurry before printing.

2.3 Design of scaffolds with electrospinning technologies

This section is dedicated to the development and processing of collagen-based systems for electrospinning technologies in order to obtain biomimetic sub-micrometric fibrous scaffolds for bone tissue engineering. The development of the collagen-based systems with particular focus on the preservation of the protein structure is reported alongside the optimisation of the electrospinning process to obtain homogeneous nanofibrous matrices, as summarised in Figure 2.1.

All the research activities have been conducted in collaboration with the Institute for Technology-Inspired Regenerative Medicine (MERLN) in the frame of the GIOTTO European Project (NMBP-22-2018; Active aGeIng and Osteoporosis: The next challenge for smarT nanobiOmaterials and 3D technologies, GA 814410).

2.3.1 Development of collagen-based formulations

Collagen solutions (COL_AA/EA/H₂O; COL_40%AA)

For the development of systems suitable for electrospinning, bovine type I collagen from Achilles tendon powders (COL) (Kensley Nash, USA) were dissolved at different concentrations ranging between 12 wt% and 25 wt% in two different solvents: a solution of acetic acid/ethyl acetate/water (AA/EA/H₂O) with a ratio of 40:30:30 and a solution of 40% acetic acid in water (AA).

The selection of collagen concentrations and solvents were based on previous studies reported in the literature and aimed at preserving the structural properties of collagen, avoiding its denaturation [28,29].

To promote the proper dissolution of collagen powders considering the high concentrations, the solutions were stirred at 4 °C overnight and used immediately after preparation.

MBG_SG_Sr4% containing systems (COL_AA/MBG_SG_Sr4%)

After optimisation of collagen concentration and solvent, nanoparticles of mesoporous bioactive glasses (MBG_SG_Sr4%) prepared as described in Section 2.2.1 were selected to obtain bioactive hybrid formulations. After complete dissolution of collagen powders in 40% acetic acid at 4 °C, MBG_SG_Sr4% nanoparticles were dispersed in the same solvent with a horn sonicator for 6 minutes and subsequently incorporated into the collagen solution to obtain final concentrations of 5 wt%. The resulting suspension was stirred for 1 hour at room temperature to obtain a homogeneous system and immediately used for electrospinning tests.

2.3.2 Assessment of potential collagen denaturation by Circular Dichroism (CD) and Attenuated Total Reflection Fourier Transform Infrared Microscopy (ATR-FTIR)

Circular Dichroism (CD)

Circular dichroism, performed in collaboration with Università degli Studi di Torino, was exploited to investigate the preservation of the secondary structure of collagen upon treatment with the different solvents and electrospinning conditions. Analyses were performed with a JASCO J-815 Circular Dichroism Spectropolarimeter, equipped with a Xe arc lamp, to record data in the far-UV spectral range. CD spectra resulted from the average of 3 scans recorded for each sample at 50 nm/sec scanning rate. The measurements were carried out using a quartz circular cuvette with a path length of 0.1 mm in the 180-260 nm wavelength range. The different samples were prepared using a concentration of collagen of 0.1 mg/mL in distilled water.

All spectra were corrected after acquisition using the correspondent solvent medium as baseline (distilled water) and analysed at room temperature (20 °C).

CD spectra of non-treated type I collagen powders were used as control and compared with lyophilised collagen reconstituted after dissolution in AA/EA/H₂O and 40%AA in addition to the collected electrospun matrix of 25%COL_40%AA.

Attenuated Total Reflection - Fourier Transform Infrared Microscopy (ATR-FTIR) Spectroscopy

FTIR analysis in the attenuated total reflection mode (ATR) was used for the chemical characterisation of the electrospun scaffolds.

FTIR spectra, in the 4000–500 cm⁻¹ range, were collected in the transmittance mode by using a Perkin Elmer Spectrum 1000 spectrometer, at a spectral resolution of 4 cm⁻¹ and accumulation of 32 scans, by using the attenuated total reflection (ATR) mode.

The ATR-FTIR spectra obtained from the non-treated dry bovine collagen powders were compared with those obtained from 25%COL_40%AA and 25%COL_40%AA/MBG_SG_Sr4% electrospun scaffolds produced as described in Section 2.3.4. For the analysis, the electrospun scaffolds were dried overnight, peeled from the aluminium foil, and subsequently used for measurement.

2.3.3 Rheology of the collagen systems

All the rheological tests were performed using a DHR-2 controlled stress rotational Rheometer (TA Instruments, Waters) equipped with a parallel plate geometry with a diameter of 20 mm and a Peltier plate system to constantly control the system temperature as described in Section 2.2.11.

Flow ramp tests at 23 °C were conducted observing the variation of the system viscosity over a wide range of shear rates (0.01 – 1000 s⁻¹).

Flow ramp tests were performed on the following formulations: 12%COL_AA/EA/H₂O; 20%COL_AA/EA/H₂O; 25%COL_40%AA; 25%COL_40%AA/5%MBG_SG_Sr4%.

2.3.4 Electrospinning tests

Electrospinning is a system able to exploit the formation of electrostatic forces, upon application of an electric field, to produce sub-millimetric and sub-micrometric fibres starting from the extrusion of polymeric solutions. During the process, the material is extruded by an emitter at specific flow rates and drawn to the collector due to the application of high voltages. Once the surface charge exceeds the material surface tension, a Taylor cone is created, and the polymeric jet is attracted to the collector. The process conditions cause the evaporation of the solvent and the deposition of dry fibres with dimensions according to the set parameters and the physico-chemical features of the material. Moreover, different types of collectors can be used to obtain different orientations of the fibres upon deposition.

The electrospinning tests were performed using a custom-made electrospinning setup placed under a chemical hood and equipped with a temperature and humidity control system. A plate collector and an emitter of 21 G (0.8 mm internal diameter) were used for all the tests in order to obtain randomly oriented fibrous mats, as schematically represented in Figure 2.11.

The different collagen-based formulations tested were prepared as described in Section 2.3.1 and left 30 minutes at room temperature before loading into 5 mL syringes. Before starting the process, aluminium foil was wrapped on the plate collector to promote the easier removal of the electrospun matrix.

Electrospinning tests were conducted at a distance between emitter and collector of 10-15 cm with voltage and flow rate ranging from 18 to 20 kV and 150 to 50 μ L/h respectively and keeping a constant temperature of 23 °C at 40% humidity. For each formulation, the process was carried out for 20 minutes testing different process parameters, observing the formation of a stable Taylor cone upon application of the selected voltage.

The resulting electrospun matrices were removed from the collector and left to dry overnight at room temperature before analysis.

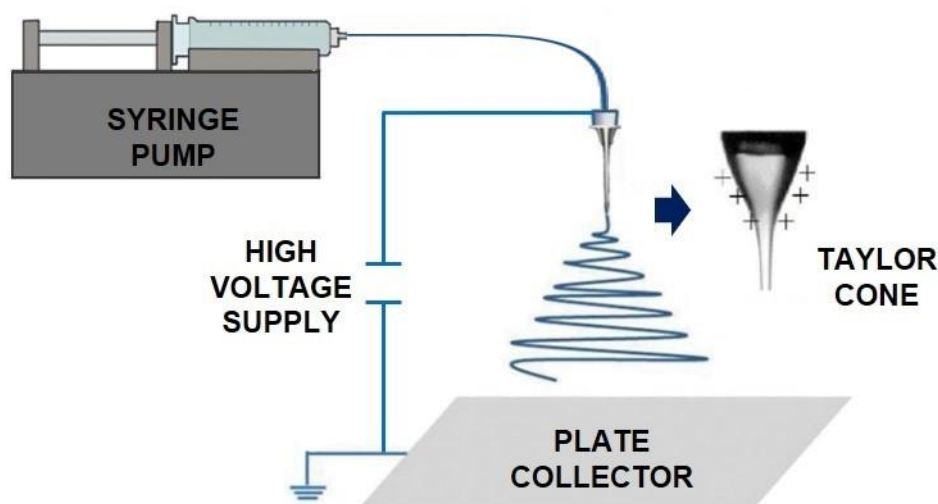


Figure 2. 11 Schematic representation of the electrospinning process.

2.3.5 Morphological assessment by Scanning Electron Microscopy

For investigation of fibre diameter and morphology as well as particle distribution, samples of electrospun matrices obtained with different process parameters as described in Section 2.3.4 were attached onto 12 mm disks and gold coated using a Cressington Sputter Coated 180 auto. The prepared samples were subsequently analysed by means of Environmental Scanning Electron Microscopy (ESEM) using a Philips XL-30 ESEM instrument (Philips, and Versa 3D, FEI) at 10 kV in order to define their morphological features.

ESEM images were acquired for all the electrospun matrices obtained by the processing of the following formulations: 12%COL_AA/EA/H₂O;
20%COL_AA/EA/H₂O; 25%COL_40%AA;
25%COL_40%AA/5%MBG_SG_Sr4%.

2.4 In vitro biological assessment

Part of the work described in this section has been previously published [2,4].

The *in vitro* biological assessment of all the designed systems was performed in collaboration with Istituto Ortopedico Rizzoli of Bologna (Scienze e Tecnologie Biomediche, IRCCS) and Università Politecnica delle Marche (Department of Clinical and Molecular Sciences – DISCLIMO) as Third Parties in the ERC BOOST Project (Biomimetic trick to re-balance Osteoblast-Osteoclast loop in osteoporosis treatment: a Topological and materials driven approach, GA 681798).

A preliminary evaluation of the cytocompatibility of materials and crosslinking methods was performed using single cultures of pre-osteoblasts (i.e., MG-63 and Saos-2 cells) [2,4].

Once optimised, the composite systems and scaffolds were tested in presence of co-cultures of osteoblasts (OBs) and osteoclasts (OCs), observing viability, proliferation and maturation of OBs derived from human trabecular bone and peripheral blood mononuclear cells (PBMCs) isolated from fresh buffy coat samples. In details, the protocol for OB and OC co-culture was developed and optimised by Istituto Ortopedico Rizzoli and subsequently used in presence of the developed biomaterials and scaffolds.

For the biological study, all the material samples were prepared in sterile conditions at Politecnico di Torino, while cell cultures and tests were performed at Istituto Ortopedico Rizzoli and Università Politecnica delle Marche, after previous agreement of all the protocols for the different analyses. The morphological assessment of the collected samples was carried out at Politecnico di Torino by using Scanning Electron Microscopy (SEM).

2.4.1 Cell culture protocols

Cell culture and experiments using Saos-2 pre-osteoblastic cells were carried out by Università Politecnica delle Marche, while the MG-63 cell culture and the experiments in presence of OB and OC co-cultures were performed by Istituto Ortopedico Rizzoli.

MG-63 Cell Culture

MG-63 human osteosarcoma cells (ATCC CRL-1427) were obtained from Flow Laboratories. The cells were maintained in a standard medium composed of Minimum Essential Medium Eagle-Alpha Modification (α -MEM) (Sigma, M0644), 1% penicillin–streptomycin (Euroclone, ECB3001D) (penicillin 10.000 Units/mL, and streptomycin 10 mg/mL) and 10% fetal bovine serum (FBS) (Sigma, F7524) in a humidified incubator at 37 °C, 95% air and 5% CO₂. For passaging trypsin/EDTA (Trypsin 0.05%–EDTA 0.02% in PBS, Euroclone, ECB3052D) was used.

Saos-2 Cell Culture

Saos-2 human osteosarcoma cells (ATCC HTB-8) were purchased from ATCC. Cells were maintained in high/low glucose (1:1) Dulbecco Modified Eagle's Medium (H-DMEM, Sigma-Aldrich, D6429) (L-DMEM, Euroclone, ECM0070L), 1% penicillin–streptomycin (Thermo Fisher Scientific, 15140122) and 10% FBS (Corning Inc., 35-079-CV), without any additional supplement in a humidified incubator at 37 °C, 95% air and 5% CO₂. For passaging trypsin/EDTA (trypsin 0.05%–EDTA 0.02% in PBS, Sigma-Aldrich, T4174) was used.

2.4.2 Cell seeding on bulk biomaterial samples

To perform preliminary cytocompatibility tests, thin biomaterial layers were obtained by deposition of 200 μ L of suspension (prepared in sterile conditions) in each well of 48-well plates (well surface area $\sim$ 0.95 cm²). The plates were incubated

for 3 h at 37 °C and 95% humidity, to promote the sol–gel transition of the system. To test the cytocompatibility of the chemical treatments, samples were chemically crosslinked with 1 mL of the specific crosslinking solution (1% 4-StarPEG, 0.5% GEN/PBS and 0.5% GEN/EtOH) incubating at 37 °C, 95% humidity for 24 h. To remove any residual of crosslinker, two washing steps with PBS were performed by placing the crosslinked samples in an orbital shaking for 30 min at 37 °C, 95% humidity. Before cell seeding, 2 mL of culture medium were added in each well, to pre-wet the material for 2 h at 37 °C and 95% humidity.

Cells at 80% of confluence were trypsinized, and MG-63 cells (2×10^5 /well) and Saos-2 cells (0.5×10^5 /well) were seeded on biomaterial samples, while cells plated at the bottom of tissue culture polystyrene (TCPS) wells were used as controls. The plates were incubated at 37 °C, 95% humidity, and cell adhesion, viability and morphology were assayed at predefined time points (24 h, 72 h).

2.4.3 Indirect co-culture of OBs and OCs

To perform the co-culture tests on GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA, material samples were created by incubating 400 μ L of suspension (prepared in sterile conditions) in each well of 24-well plates at 37 °C for 3 hours, subsequently chemically crosslinked with 2 mL of 0.5% genipin in 70% ethanol solution. After 24 hours at 37 °C, the crosslinker solution was removed and the samples were washed two times with PBS by placing the crosslinked samples in an orbital shaking for 30 min at 37 °C, 95% humidity. Before cell seeding, 2 mL of culture medium were added in each well, to pre-wet the material for 2 h at 37 °C and 95% humidity. Culture medium, i.e. basal medium (BM), consisted of 1:1 Dulbecco's Modified Eagle Medium high glucose supplemented with of 10% foetal bovine serum and 1% penicillin-streptomycin (DMEM-HG) and Dulbecco's Modified Eagle Medium low glucose supplemented with of 10% foetal bovine serum and 1% penicillin-streptomycin (DMEM-LG). After the material preparation, cell seeding was performed using an indirect contact system, as schematically represented in Figure 2.12A. In details, OBs were seeded onto the surface of the material layer at a density of $5 \times 10^4/\text{cm}^2$ in complete DMEM-LG, replaced after 4 hours with basal medium. In parallel, PBMCs (OC-precursors) were isolated from fresh buffy coat and seeded at a density of $3 \times 10^6/\text{cm}^2$ into a transwell device to allow the exchange of paracrine signalling, obtaining a final OB/PBMC ratio of 1/20.

The co-culture systems were maintained at 37 °C, 95% humidity and carried out up to 21 days, where at predefined time points (24 h, 7d, 14d, 21 d) the viability, proliferation and differentiation of both OBs and OCs was assessed.

Similarly, 3D printed scaffolds of Coll/MBG_SG_Sr4%/nano-HA crosslinked in GEN/EtOH (prepared as described in Section 2.2.12) were tested in OB and OC co-culture systems as illustrated in Figure 2.12B. Before seeding the cells, scaffolds were pre-wetted by incubating in 2 mL of complete culture medium (basal medium, BM) for 3 hours. Subsequently, $5 \times 10^4/\text{cm}^2$ OBs were resuspend in 40 μ L of

Dulbecco's Modified Eagle's Medium low glucose (DMEM-LG) and seeded onto the upper side of the mesh-like scaffolds while $1 \times 10^6/\text{cm}^2$ PBMCs, freshly isolated from buffy coat sample, were previously seeded into the transwell placed in indirect co-culture with the OB/scaffold system. Viability, proliferation and differentiation of OB and OC cells were assessed at 24h, 7d, 14 d and 21d.

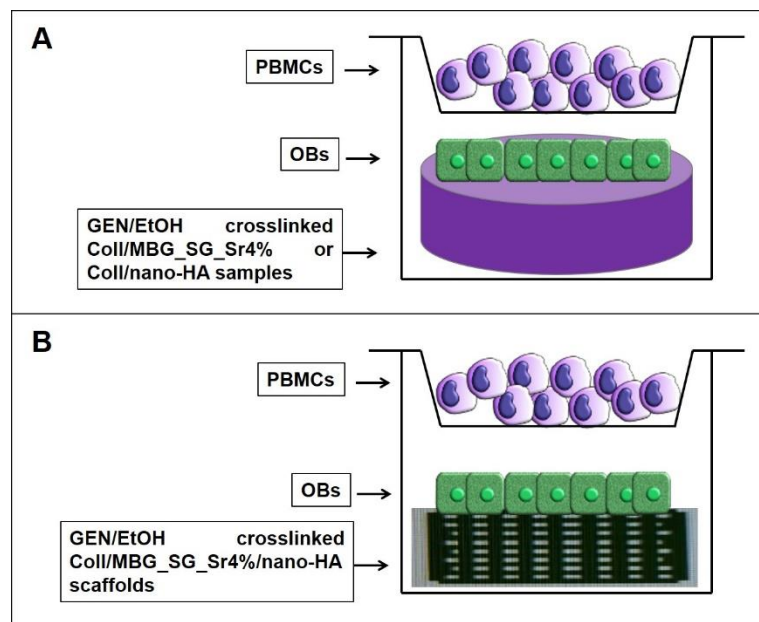


Figure 2. 12 Schematic representation of OB and OC co-culture on bulk samples of GEN/EtOH crosslinked Coll/MBG_SG_Sr4% or Coll/nano-HA (A) and GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA scaffolds (B).

2.4.4 Cell viability by means of Alamar Blue Assay

To assess the presence of metabolically active cells seeded onto the different material samples, the one-step Alamar Blue assay (Invitrogen, DAL1100) was performed according to the manufacturer's instructions. Alamar blue assay is a one-step test based on the use of a fluorogenic redox indicator which quantitatively estimates the growing cells in culture. This dark blue dye becomes red when processed by viable cells, due to its conversion in a reduced and highly fluorescent form. The quantity of reduced fluorescent dye, evaluated by means of a spectrophotometer, is considered a direct indicator of cells viability.

For the analysis, at predefined time steps, the culture medium was removed, replaced with the Alamar Blue solution prepared as 10% v/v in fresh cell culture medium and incubated at 37 °C, 95% humidity for 4 h. Then the fluorescence of Alamar Blue solution was quantified by using a microplate reader (Infinite F200 PRO, TECAN) at 535 nm excitation and 590 nm emission wavelengths. Data are expressed as a percentage of control cultures (only cells seeded in tissue culture plates) and reported as mean value $\pm$ standard deviation of triplicates. The material without cells was also analysed and considered as background.

Table 2.4 indicate cell types, material samples and time points considered for the cell viability assay.

Table 2. 4 Summary of the samples tested for cell viability by Alamar Blue assay.

CELL TYPE	SAMPLES	TIME POINTS
MG-63 cell line (OBs)	• Coll/MBG_SG_Sr4%; • 4-StarPEG crosslinked Coll/MBG_SG_Sr4%; • GEN/PBS crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	24h, 72h
Saos-2 cell line (OBs)	• GEN/PBS crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	24h, 72h
Human OB and OC co-culture	• GEN/EtOH crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/nano-HA • 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	24h, 7d, 14d, 21d

2.4.5 Cell adhesion by means of Scanning Electron Microscopy (SEM)

To investigate the morphology and cell adhesion on the different systems tested, Scanning Electron Microscopy analyses were exploited, where images were acquired by using a Desktop SEM Phenom XL (Phenom-World B.V., The Netherlands) at an accelerating voltage of 15 kV.

Cell-seeded samples for morphological analysis were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma, C-0250), followed by washing in PBS and post-fixation in 1% osmium tetroxide (Electron Microscopy Sciences, 12310) in 0.1 M sodium cacodylate buffer. After complete dehydration achieved by graded alcohol series and hexamethyldisilane, the resulting dry samples were sputter coated with a 7 nm thick platinum layer.

Table 2.5 indicate cell types, material samples and time points considered for the cell adhesion assessment by SEM.

Table 2. 5 Summary of the samples assessed for cell-adhesion by SEM.

CELL TYPE	SAMPLES	TIME POINTS
MG-63 cell line (OBs)	• Coll/MBG_SG_Sr4%; • 4-StarPEG crosslinked Coll/MBG_SG_Sr4%; • GEN/PBS crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	24h, 72h
Saos-2 cell line (OBs)	• GEN/PBS crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	24h, 72h
Human OBs (OB/OC co-culture systems)	• GEN/EtOH crosslinked Coll/MBG_SG_Sr4%; • GEN/EtOH crosslinked Coll/nano-HA •	7d, 14d, 21d

2.4.6 Assessment of cell maturation in OB/OC co-culture

Live Dead Assay

Live Dead assay (LIVE/DEAD™ Cell Imaging Kit (488/570), R37601, Invitrogen) was performed to visualize viable OBs seeded onto biomaterial bulk samples (GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA) and 3D printed scaffolds (GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA) as a further proof of cell viability.

The experiment enabled the staining of OB cells onto biomaterial samples with calcein-AM (green reagent for viable cells), thus observing both OB morphology and vitality.

At predefined time points (24h, 7d, 14d, 21d), cell culture medium was removed, and the samples were washed with PBS. Following manufacturer's instructions, the staining solution was prepared and further incubated with cell seeded samples for 20 minutes at 37°C in orbital shaking and dark conditions. After washing with PBS, samples were observed under fluorescent microscope and representative images were acquired at different magnification.

Tartrate-resistant acid phosphatase (TRAP), Phalloidin-FITC and Hoechst 33258 stain

TRAP and Phalloidin-FITC stains, alongside the assessment of cell multinuclearity (Hoechst 33258) were performed onto the PBMC/OC-precursors seeded into the transwell and maintained in indirect co-culture with OB/material samples. The different stains were performed at 7 and 14 days as proof of differentiation for osteoclast function.

TRAP stains tartrate-resistant acid phosphatase in osteoclasts and it is used as histochemical marker since it represents a glycosylated monomeric enzyme highly expressed by mature OCs.

For the analysis, at the predefined time points, cells were fixed in 3% paraformaldehyde and 2% sucrose in PBS and subsequently exposed to HEPES-Triton solution for 5 minutes in order to allow cell membrane permeabilization. After washing with PBS, cells were immersed in the TRAP staining solution (Acid Phosphatase Leukocyte assay, Sigma, 387A), maintained for 1 hour at 37 ° C in dark conditions.

Phalloidin-FITC is a green, fluorescent dye used to stain actin filaments, that enables the morphological assessment of differentiating PBMCs.

For the experiments, cells were fixed with 3.7% paraformaldehyde in PBS, and the membranes were permeabilized using 0.5% TritonX in PBS for 10 minutes. The cell monolayer was subsequently treated with Phalloidin-FITC staining solution in PBS for 45 minutes in dark conditions.

Hoechst 33258 (bis-benzimide) nuclear acid stain is a blue, fluorescent cell-permeant dye used to observe the nuclei thanks to its ability to emit blue fluorescence when bound to dsDNA. Hoechst was applied as last fluorescent labelling, after TRAP or Phalloidin staining.

In detail, after obtaining cell membrane permeabilization using HEPES-Triton solution for 5 minutes, cells were washed and exposed to 1.25 µg/ml Hoechst 33258 solution for 10 minutes, keeping dark conditions.

2.4.7 Statistical analyses

Statistical analyses regarding the differences between the experimental groups and between the two time-endpoints were accomplished by using the nonparametric Mann–Whitney test for unpaired data, using the StatView 5.01 for Windows software (SAS Institute Inc., Cary, NC, USA). Results were considered as mean and transformed into a percentage of viability. Only $p < 0.05$ was considered statistically significant. The p-value was at the margin of statistical significance, since it was attested at 0.0495.

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CHAPTER 3

Design and characterisation of collagen-based hybrid formulations for 3D extrusion printing: Results and Discussion

3.1 Introduction

Type I collagen, strontium containing mesoporous bioactive glasses (Sr-MBGs) and hydroxyapatite nanoparticles were combined to develop bioactive collagen-based systems suitable for the 3D extrusion printing of scaffolds for bone tissue engineering.

Type I collagen and hydroxyapatite represent the main organic and inorganic phase of bone tissue respectively, while strontium enriched MBGs are well known for their high bioactive and pro-osteogenic features. Consequently, the synergistic effect of these materials can be exploited to design biomimetic and bioactive systems to boost the bone tissue regeneration particularly considering pathological conditions.

This chapter will be thus focused on the protocols developed for different collagen-based hybrid formulations and the resulting properties of the systems before and after their sol-gel transition in physiological conditions of temperature and pH (37 °C, pH 7.4). Moreover, the use of different chemical crosslinking methods used to increase the mechanical stability of the collagen-based composites will be reported. The presented results enabled the selection of three optimised formulations and the most promising crosslinking strategy to be used for the design of bone-like scaffolds by means of extrusion printing technologies.

Firstly, based on the expertise of the research group on the synthesis of mesoporous bioactive glasses, micro- and nano-sized Sr-MBGs produced by means of two different synthesis methods, were incorporated in high-concentrated collagen solutions to create homogeneous systems, and the influence of collagen concentration as well as particle size over the final material properties was investigated. The comprehensive characterisation of the systems in terms of morphological, physico-chemical, bioactive, and rheological features enabled the selection of the most promising Sr-MBG containing formulation, as described in Section 3.2. The suitability of the developed systems as biomaterial inks was initially assessed by rheological analyses while the ability of releasing osteogenic strontium ions was investigated, particularly comparing the resulting release kinetics with those observed for the inorganic matrices alone.

After the optimisation of the synthesis process, the resulting rod-like nano-HA particles were used to develop a collagen-based biomimetic hybrid formulation for 3D extrusion printing applications. Considering the tendency of nano-HA to form agglomerates, the use of a cytocompatible dispersing agent (Darvan 821-A) was explored, optimising the final concentration in the material formulation as reported in Section 3.3. The most promising system was selected by performing a comprehensive study in terms of morphological, physico-chemical and rheological features, with particular focus on the potential printability.

Based on the results obtained from the combination of type I collagen with the single inorganic phases (Sr-MBGs and nano-HA), an additional hybrid formulation was optimised combining both nano-sized Sr-MBGs and nano-HA, as presented in Section 3.4. Similarly, the developed system was characterised observing its morphological, physico-chemical, and bioactive properties while its use as biomaterial ink was explored by means of a comprehensive rheological study.

Based on the poor mechanical and thermal stability demonstrated by the collagen-based matrices, different cytocompatible chemical crosslinkers of collagen were tested and the results are reported in Section 3.5. In particular, the chemical crosslinking strategies were firstly tested on the nano-size Sr-MBG containing system (Coll/MBG_SG_Sr4%), as it represented the first optimised formulation.

According to the literature, 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) combined with N-hydroxysuccinimide (NHS), 4-arm polyethylene glycol succinimidyl glutarate (4-StarPEG) and genipin are among the most used cytocompatible chemical crosslinkers of collagen. They are able to enhance the mechanical features and stability of the collagenous matrices by promoting the formation of inter and intra-molecular covalent bonds.

Considering that genipin reported the best results in terms of crosslinking efficiency, the optimised chemical treatment was exploited to increase the properties of the other developed systems.

3.2 Design and characterisation of Sr-MBG containing collagen-based formulations (Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%)

Part of the work described in this chapter has been previously published during this PhD thesis [1–4].

This section will describe the development and characterisation of hybrid formulations combining type I collagen and strontium containing bioactive glasses (Sr-MBGs), particularly exploring their physico-chemical features and their potential use as bioactive biomaterial inks for extrusion printing applications.

The combination of type I collagen with bioactive glasses have been widely reported to be promising in the design of bioactive systems for bone tissue engineering [5–9]. Among the large variety of bioactive glasses in terms of morphology, size and composition, this study was focused on the use of strontium enriched MBGs particles presenting a spherical morphology and different size for the development of printable bioactive collagen-based systems. Sr-MBGs have been selected as promising candidates due to their significant bioactive and pro-osteogenic properties recently reported by different studies [10–12].

In detail, two different size of Sr-MBGs and collagen concentrations were investigated in order to select the most suitable system for the further design of biomimetic bioactive scaffolds.

As already reported in Section 2.2.1, micro- and nano-sized MBGs containing 4% molar strontium were synthesised exploiting two different methods and following the protocols developed and optimised based on previous studies and the expertise of our research group [10,13]. The incorporation of different concentrations of strontium (1%, 2%, 4% molar) in the glass formulation was previously studied by our research group and the 4% molar concentration was selected for this study in order to maximise the total amount of ion released while ensuring no cytotoxic effects [10,12]. In this frame, different works have proved that Sr-containing bioactive glass nanoparticles were able not only to influence osteoblastic cell activity but also to guide the osteogenic differentiation of bone marrow derived human stem cells. Moreover, concentrations higher than 4% mol. did not lead to additional benefit in terms of cell activity [17].

In this work, an aerosol-assisted approach was exploited to produce SiO₂-CaO-based MBG containing strontium (4 % mol.), named as MBG_SD_Sr4%, showing spherical morphology with micrometric size between 0.5 and 5 μ m, specific surface area (SSA) of about 150 m²/g and average pore diameter of about 7 nm, as illustrated in Figure 3.1A.

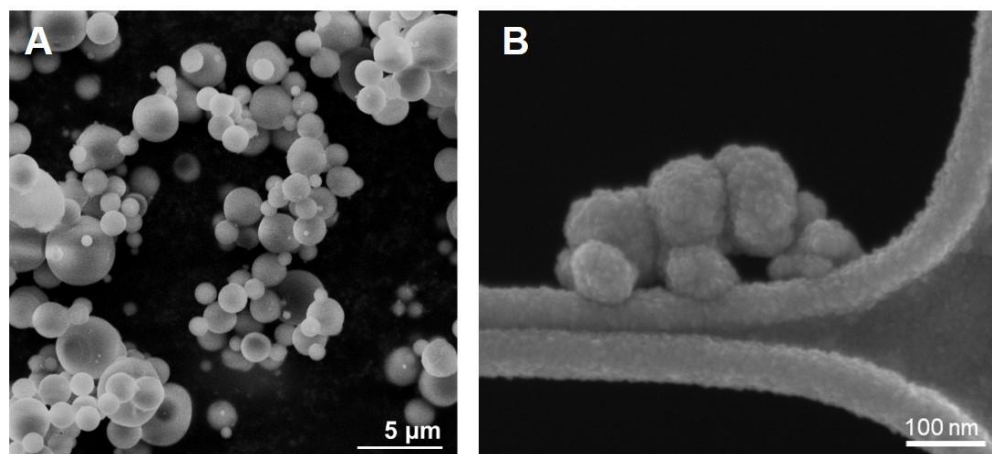


Figure 3. 1 FESEM images of MBG_SD_Sr4% (A) and MBG_SG_Sr4% (B).

On the contrary, a base-catalysed sol-gel route was used to create 4% mol. strontium containing MBGs (MBG_SG_Sr4%), based on SiO₂ and CaO, with spherical morphology and size ranging from 100 to 200 nm, higher SSA of about 550 m²/g and average pore diameter of about 4 nm (Figure 3.1B). The main features

of micro- and nano-sized strontium containing MBGs have been summarised in Table 3.1.

Compared to micro-sized particles, some studies have proven that the use of nano-sized particles can lead to improved osteoconductive properties and stiffness of the final construct due to the enhanced bioreactivity and better embedding quality [5].

Table 3. 1 Morphological and structural features of MBG_SD_Sr4% and MBG_SG_Sr4%.

Sr-MBGs	SYNTHESIS ROUTE	SIZE	SPECIFIC SURFACE AREA	AVERAGE PORE DIAMETER
MBG_SD_Sr4%	Spray drying assisted technique	0.5 – 5 μm	150 m^2/g	7 nm
MBG_SG_Sr4%	Base-catalysed sol-gel method	100 -300 nm	500 m^2/g	4 nm

According with different studies in the literature and with the aim to obtain high fibrillar densities, collagen concentrations of 1 wt% and 1.5 wt% were dissolved in 0.5 M acetic acid solutions to obtain the final hybrid suspensions [14–16]. The use of concentrations higher than 1.5 wt% were found to hinder the proper dissolution of collagen molecules as well as the creation of homogeneous neutralised suspensions. Based on this, higher collagen concentrations were not considered suitable for the targeted material formulation.

Starting from the ratio between organic and inorganic phase present in the natural bone tissue (53% vol. of collagen and 47% vol. of inorganic phase) [17], the correspondent weight amounts were calculated considering the specific densities of collagen (about 1.4 g/cm^3), MBG_SG_Sr4% (about 1 g/cm^3) and MBG_SD_Sr4% (about 2 g/cm^3) particles [18]. In details, 61.8 wt% of type I collagen powders were combined with 38.2 wt% of MBG_SG_Sr4% while 47.2 wt% of type I collagen was mixed with 52.8 wt% of MBG_SD_Sr4% to create the two different hybrid formulations [17–19].

After the synthesis, MBG_SD_Sr4% and MBG_SG_Sr4% were resuspended in 0.5 M acetic acid before addition into the collagen solution. To obtain a proper dispersion of the particles and considering the different tendency to form aggregates due to their size, MBG_SD_Sr4% and MBG_SG_Sr4% suspensions were sonicated for 30 minutes and 1 hour respectively, until all particle aggregates were disrupted.

The resulting inorganic phase suspensions were then homogeneously mixed with type I collagen solutions to form two different collagen-based suspensions at 4 °C that were subsequently neutralized to promote the formation of collagen fibres in physiological conditions. The final suspension containing micro-sized MBGs will be hereafter referred as Coll/MBG_SD_Sr4%. On the contrary, the MBG_SG_Sr4% containing system will be reported as Coll/MBG_SG_Sr4%.

A comprehensive rheological study was conducted to assess the potential printability of the developed systems as well as investigate the influence of collagen

concentration and particle size over the final visco-elastic properties. The results enabled the selection of the most promising material formulations that were further investigated in terms of morphological, physico-chemical and bioactive features.

3.2.1 Rheology of Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%

Rheological analyses are normally conducted to study the visco-elastic properties of the material by measuring the visco-elastic response of samples subjected to different stresses and strains. Rheological features are considered important physico-chemical factors that largely influence the material printability [20].

The overall rheological properties of the developed materials were thus explored considering the final application as biomaterial inks, applying stress conditions typically occurring during the printing process.

Considering an extrusion-based system, the biomaterial ink is rapidly subjected to significantly different stress conditions: 1) an initial resting state when stored in the cartridge; 2) high shear rates when passing through the nozzle; 3) almost zero-shear conditions once deposited, requiring the fast recovery of the material structure (*shear recovery*) [20–23]. These transitions can be clearly described by parameters such as viscosity, viscoelastic modulus and shear elastic recovery [20,24].

High values of viscosity may hinder the proper extrusion of the materials especially when using small needle diameters. For this reason, biomaterial inks showing *shear thinning* behaviour (decrease of viscosity at increasing shear rates) are considered ideal for extrusion printing technologies. In addition, high yield stress values as well as fast crosslinking kinetics can favour the stability of the material once extruded, enhancing the final shape fidelity of the printed structures [23,24]. All these factors are dependent on the physico-chemical properties of the material, where viscosity is directly linked to the specific molecular weight and concentration of the polymeric phase.

In this frame, the hybrid formulations were analysed defining the main differences observed between the systems containing micro- and nano-sized Sr-MBGs particles in addition to the influence of the collagen concentration. In particular, the rheological properties of 1 wt% and 1.5 wt% collagen solutions (pH 7.4) were measured and compared to those reported by the hybrid formulations in order to define the specific variations induced by the inorganic phase addition.

The shear stress-induced change in the material viscosity and yield stress values were studied by performing flow ramps at 10 °C, while the peculiar sol-gel transition of the systems at 37 °C was investigated by means of time sweep tests up to 1 hour.

Figure 3.2 (A, B, C) show the variation of viscosity of 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions over a wide range of increasing shear rates (10^{-2} - 10^3 s $^{-1}$). On the contrary, the yield stress values were obtained by measuring the point of inflection in the graph reporting shear rate pattern as a function of stress variation, as presented in Figure 3.2 (D, E, F). All the viscosity and yield stress values have been summarised in Table 3.2.

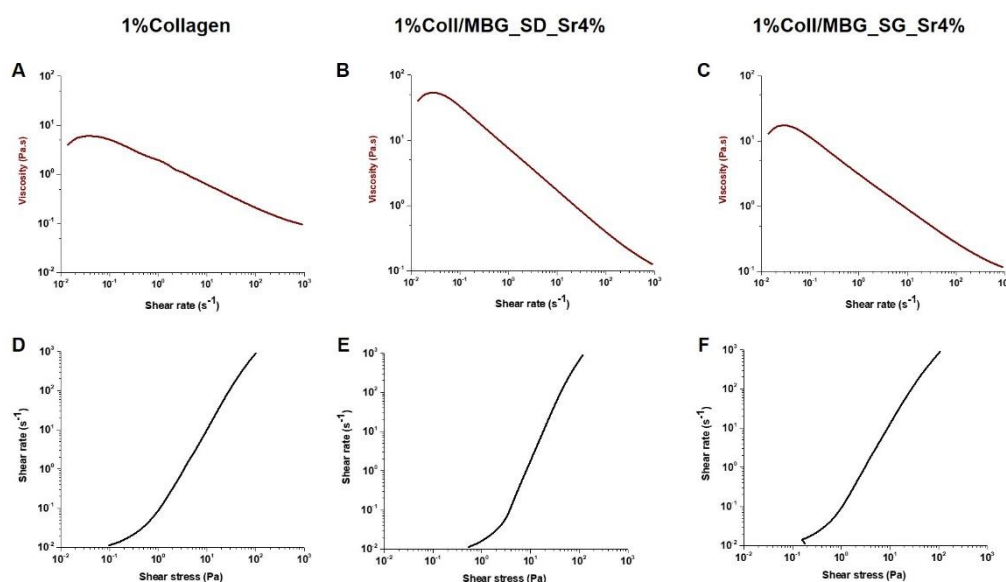


Figure 3. 2 Flow ramps performed on 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions at 10 °C showing viscosity variation with increasing shear rate values (A, B, C) and the shear rate pattern as a function of stress variation (D, E, F).

According to previous studies in the literature [22,25,26], data proved the pseudo-plasticity (shear thinning) of the collagen solution and suspensions given by the rapid decrease of the material viscosity with increasing shear rates. In detail, starting from about 6.03 Pa.s at zero-shear conditions, the viscosity value decreased up to about 0.1 Pa.s at 10^3 s $^{-1}$ for 1 wt% collagen solution.

Table 3. 2 Summary of viscosity and yield stress values of 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions at 10 °C.

	Viscosity η (max)	Viscosity η (min)	Yield stress
1 wt% Collagen	6.1 Pa.s	0.1 Pa.s	1.2 Pa
1%Coll/MBG_SD_Sr4%	53.7 Pa.s	0.1 Pa.s	2.8 Pa
1%Coll/MBG_SG_Sr4%	17.2 Pa.s	0.1 Pa.s	1.5 Pa

As reported in Table 3.2, when MBG_SD_Sr4% and MBG_SG_Sr4% particles were added to the 1 wt% collagen solution, a slight increase of viscosity and yield stress values were registered. Therefore, the reported data proved the preservation of the marked pseudo-plastic behaviour of collagen, after addition of both micro- and nanoparticles of Sr-MBGs. In detail, higher values of viscosity at low shear

rates were registered for the MBG_SD_Sr4% containing systems, reporting values of about 53.7 Pa.s compared to only 17.2 Pa.s measured for the 1%Coll/MBG_SG_Sr4% system. In the case of suspensions, the shear-thinning behaviour and the viscosity values are strongly dependent on polymeric entanglements as well as potential interactions between the solid particles and the polymeric phase [20]. The slight increase of the overall rheological properties after addition of the inorganic phase suggested the formation of weak interactions between collagen and MBG particles, as confirmed in different studies reported in the literature [5,9].

The same rheological assessment was subsequently performed on the hybrid formulations containing an increased 1.5 wt% collagen concentration, as shown in Figure 3.3.

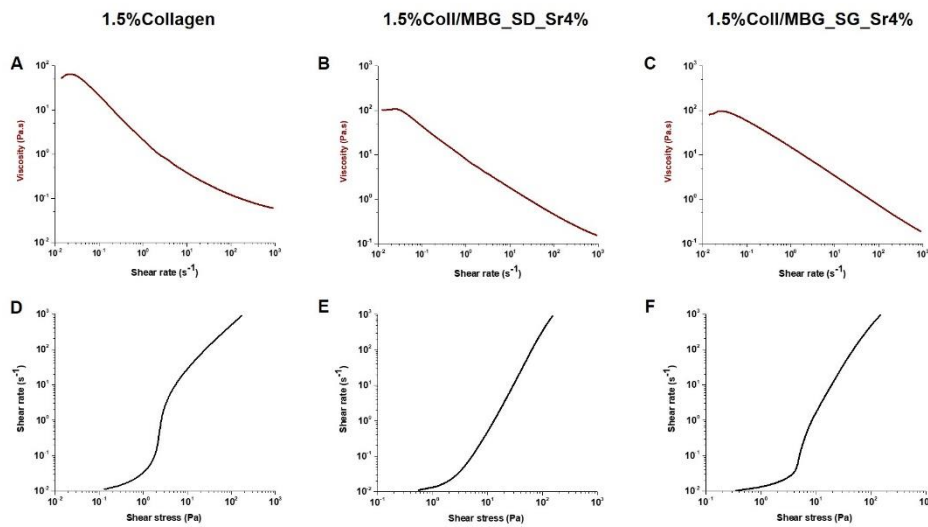


Figure 3.3 Flow ramps performed on 1.5 wt% collagen solution, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% suspensions at 10 °C showing viscosity variation with increasing shear rate values (A, B, C) and the shear rate pattern as a function of stress variation (D, E, F).

As expected, increasing polymer concentrations led to greater viscosity and yield stress values (Table 3.3) due to the higher polymeric entanglements. Upon shearing, the polymeric chains disentangle and align, favouring the overall material flow [20].

Figure 3.3 (A, B, C) confirmed the shear thinning behaviour of the hybrid formulations at 10 °C, where no evident differences in viscosity and yield stress values were observed between 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% systems. Compared to the 1.5 wt% collagen solution characterised by a viscosity of about 64.2 Pa.s, the addition of Sr-MBG particles led to the doubling of the viscosity and yield stress values of the materials, as reported in Table 3.3.

Table 3. 3 Summary of viscosity and yield stress values of 1.5 wt% collagen solution, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% suspensions at 10 °C.

	Viscosity η (max)	Viscosity η (min)	Yield stress
1.5 wt% Collagen	64.2 Pa.s	0.1 Pa.s	2.2 Pa
1.5%Coll/MBG_SD_Sr4%	108.5 Pa.s	0.1 Pa.s	4.4 Pa
1.5%Coll/MBG_SG_Sr4%	97.3 Pa.s	0.1 Pa.s	4.1 Pa

With the aim to improve the stability after extrusion, materials performing sol-gel transition in response to physical and chemical stimuli are considered among the most eligible for extrusion printing applications [22,23]. The gelation kinetics of the collagen-based systems were, thus, investigated observing the variation of G' (storage modulus) and G'' (loss modulus) at 37 °C for up to 1 h.

The visco-elastic behaviour of materials can be expressed by the storage modulus G' , defining the energy elastically stored during the deformation, and the loss modulus G'' , measuring the energy dissipated and thus linked to the viscous flow. G' and G'' are thus resulting from the number and type of crosslinks and entanglements present within the material [20,24].

Figure 3.4 report the results of the time sweep tests performed at 37 °C on 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions, plotting the variation of the visco-elastic properties (G' , G'') as a function of time.

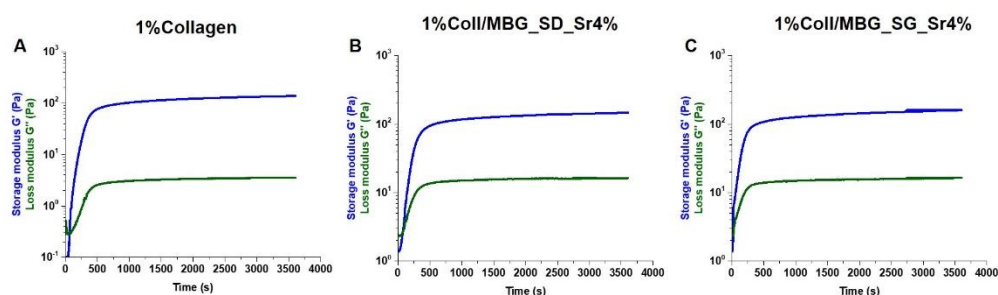


Figure 3. 4 Sol-gel transition of 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions detected through the time sweep test at 37 °C.

The sol-gel transition collagen solution and suspensions, identified by the sharp increase of G' compared to G'' , was detected after less than 100 seconds, where 1 wt% collagen solution and 1%Coll/MBG_SD_Sr4% showed faster gelation kinetics. For all the materials, a stable gel was formed after about 500 seconds, detecting a significant gap between G' and G'' . Greater values of G' compared to G'' were indicative of a major elastic response over the viscous behaviour [9]. As a result of the collagen fibril reconstitution, increasingly stronger matrices were obtained after up to 1 h at 37 °C, reporting final G' values of 138.1 Pa, 146.5 Pa and 159.9 Pa for 1 wt% collagen, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% respectively, as reported in Table 3.4.

Table 3. 4 Summary of storage modulus (G'), loss modulus (G'') and gel point registered for 1 wt% collagen solution, 1%Coll/MBG_SD_Sr4% and 1%Coll/MBG_SG_Sr4% suspensions at 37 °C.

	Storage modulus (G') after 1 hour at 37 °C	Loss modulus (G'') after 1 hour at 37 °C	Gel point
1 wt% Collagen	138.1 Pa	14.6 Pa	< 50 s
1%Coll/MBG_SD_Sr4%	146.5 Pa	16.2 Pa	< 50 s
1%Coll/MBG_SG_Sr4%	159.9 Pa	16.4 Pa	< 100 s

The rheological analyses performed on the hybrid formulations proved that the addition of both MBG_SD_Sr4% and MBG_SG_Sr4% particles did not prevent the molecular reorganisation of collagen [22,23]. Similar sol-gel transition kinetics were observed for both systems, where a slight increase of the storage modulus was registered after addition of the inorganic phase compared to the 1 wt% collagen system and a stable gel was formed after about 500 seconds at 37 °C. According to the previous observations, the reported data seem to exclude the formation of strong bonds between the organic and inorganic phase [5].

The potential achievement of stronger gel due to the higher polymeric concentration was explored on 1.5% collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4%, as illustrated in Figure 3.5 [27,28].

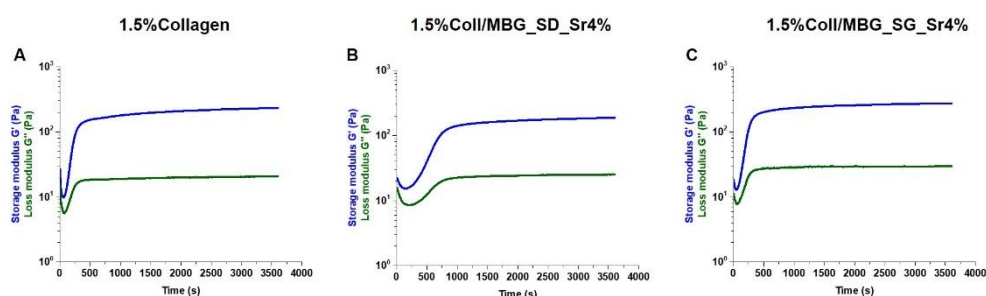


Figure 3. 5 Sol-gel transition of 1.5 wt% collagen solution, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% suspensions detected through the time sweep test at 37 °C.

As expected, higher values of storage modulus were registered when Sr-MBGs were added to 1.5 wt% collagen solutions. However, some differences were observed between the MBG_SD_Sr4% and MBG_SG_Sr4% containing systems, as also reported in Table 3.5.

In detail, the addition of micro-sized particles led to a slight shift of the gel point of the system compared to the collagen alone, showing the formation of a stable gel after about 1000 seconds. In addition, the storage modulus registered after 1 hour at 37 °C (187.6 Pa) was lower compared to the 1.5 wt% collagen system (234.2 Pa), suggesting an interfering of MBG_SD_Sr4% particles with the physical self-assembly of collagen molecules.

On the contrary, MBG_SG_Sr4% containing systems showed a fast sol-gel transition and a slight increase of the storage modulus registered at 37 °C after 1 hour, that can be ascribed to the higher reinforcing effect and the better embedding into collagen fibrils of nanosized compared to micro-sized particles, as confirmed by other studies [29]. These data also demonstrated the creation of a stable gel after less than 500 seconds.

Table 3. 5 Summary of storage modulus (G'), loss modulus (G'') and gel point registered for 1.5 wt% collagen solution, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% suspensions at 37 °C.

	Storage modulus (G') after 1 hour at 37 °C	Loss modulus (G'') after 1 hour at 37 °C	Gel point
1.5 wt% Collagen	234.2 Pa	20.7 Pa	< 50 s
1.5%Coll/MBG_SD_Sr4%	187.6 Pa	25.1 Pa	< 200 s
1.5%Coll/MBG_SG_Sr4%	275.2 Pa	29.7 Pa	< 100 s

Based on the overall enhanced rheological properties due to the use of higher concentrations, collagen at 1.5 wt% was selected for the design of the different hybrid formulations. Additional rheological tests were thus performed to investigate the visco-elastic properties of 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4%. In fact, considering the shear-thinning behaviour performed by all the tested systems, higher values of viscosities, yield stress as well as storage modulus are normally preferred to promote the shape preservation of printed structures.

Another important key factor to obtain high shape fidelity in 3D printing is represented by the transition between the fluid-like flow and the elastic shape retention [20,24]. This transition can be measured by monitoring the recovery of the material viscosity upon removal of the applied stress [20,23].

Peak hold tests were, thus, performed, observing the response of the material subjected to stress conditions similar to those occurring during the printing process. In detail, two different subsequent shear rates of 1000 s^{-1} (100 s) and 0.1 s^{-1} (300 s) were applied to the collagen systems, reproducing the extrusion and the deposition step respectively.

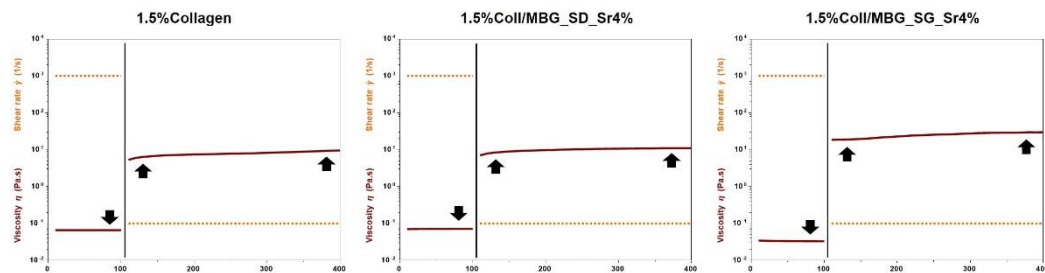


Figure 3. 6 Printing process simulation by means of the peak hold test performed at 10 °C on 1.5%Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4%.

As shown in Figure 3.6 and summarised in Table 3.6, the viscosity of all the tested systems was set to very low values of about 0.1 Pa.s as a result of the application of a constant shear rate of 1000 s^{-1} . Once the shear stress was removed (0.1 s^{-1}), viscosity immediately increased reaching plateau values of about 5.3 Pa.s, 7.1 Pa.s and 18.5 Pa.s for 1.5 wt% collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% systems respectively, showing the recovery of the material molecular structure [20]. However, after 300 seconds at low shear rate conditions, none of the collagenous systems recovered the viscosity values registered at 0.1 s^{-1} during the flow ramp test (Table 3.6). These results highlighted a time shear-dependent viscosity of the collagen-based systems, and in particular the need for a certain time to reach the original viscosity values. For this type of materials, defined as thixotropic, the extrusion process is normally more complex and requires specific strategies to improve the stability of the deposited filaments [20,24].

Table 3. 6 Viscosity values of 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% at different shear rates.

	Viscosity η (1000 s^{-1})	Viscosity η (0.1 s^{-1} - 0 s)	Viscosity η (0.1 s^{-1} - 300 s)	Viscosity η at 0.1 s^{-1} (flow ramp)
1.5 wt% Collagen	0.1 Pa.s	5.3 Pa.s	9.5 Pa.s	19.5 Pa.s
1.5%Coll/MBG_SD_Sr4%	0.1 Pa.s	7.1 Pa.s	10.9 Pa.s	42.4 Pa.s
1.5%Coll/MBG_SG_Sr4%	0.1 Pa.s	18.5 Pa.s	29.8 Pa.s	56.0 Pa.s

As described in Section 1.3, the fibril reconstitution, and the consequent sol-gel transition of collagen-based materials, is known to be affected by different parameters such as pH, ionic concentration, as well as incubation temperature and time [30].

The influence of the increased time of incubation at $37\text{ }^{\circ}\text{C}$ on the visco-elastic properties of the collagen-based formulations was studied by performing dynamic amplitude sweep tests on gel samples. In detail, 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% were allowed to gel and were kept at $37\text{ }^{\circ}\text{C}$ for 3 h before starting the analysis. Subsequently, G' and G'' values were measured varying the oscillation strain until a linear visco-elastic behaviour was detected.

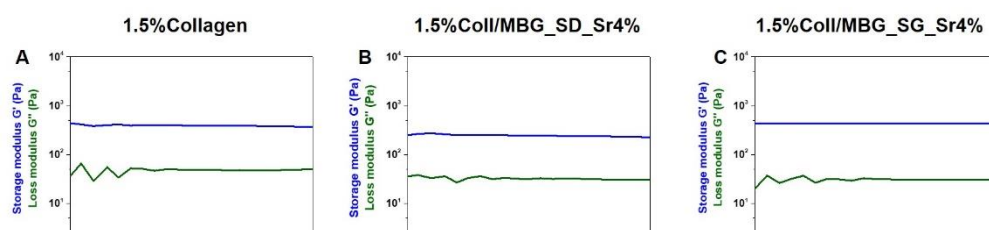


Figure 3. 7 Amplitude sweep test showing G' (storage modulus) and G'' (loss modulus) values of 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% after 3 h of incubation at $37\text{ }^{\circ}\text{C}$.

Figure 3.7 reports the pattern of G' and G'' for oscillation strains ranging from 0.01% to 1%. The large gap detected between the storage and loss moduli further confirmed the solid nature of the samples, evidenced by the significantly higher contribution of the elastic behaviour compared to the viscous properties. The averaged values of G' and G'' in the considered region were summarised in Table 3.7. Compared to values collected after 1 h, an increase of the visco-elastic modulus was detected after 3 h at 37 °C for all the systems. In particular, the highest stiffness was registered for 1.5%Coll/MBG_SG_Sr4% after 3 hours of incubation, reaching a G' value of about 434 Pa, almost twice as the modulus reported by the MBG_SD_Sr4% containing system.

The higher increase of the mechanical stiffness of collagenous matrices induced by the addition of inorganic nano-sized particles compared to micro-sized phases was already observed and reported in other studies [5]. Considering the same volume content of the two inorganic phases in the final hybrid formulation, this effect may be ascribed to the homogeneous distribution of the nanoparticles along the collagen fibrils, partially mimicking the strengthening mechanism occurring in the native ECM, together with an effective packing of collagen molecules [5,31].

Moreover, the resulting data overall suggested the relevant influence of higher gelation times on the final mechanical properties of collagenous composites, in accordance with the data found in the literature [32].

The thermal stability of the developed collagenous systems was further investigated, subjecting 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% samples to a temperature ramp between 20 and 60 °C (Figure 3.8).

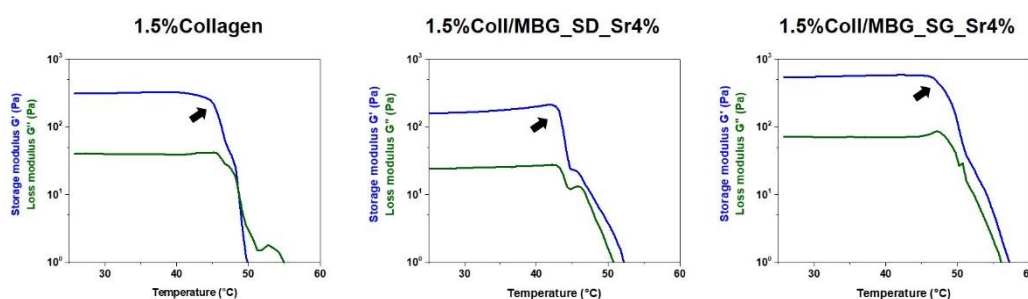


Figure 3. 8 Temperature ramp performed on 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% showing the denaturation temperature of the systems.

All the systems reported a sharp decrease of both storage and loss modulus between 41 °C and 45 °C, mainly related to the denaturation of the collagenous matrix and thus the breakage of the weak inter- and intra-molecular bonds, leading to the subsequent loss of the structural and mechanical stability of the material [16].

The slight increase of the denaturation temperature in presence of Sr-MBG particles can be ascribed by the potential presence of weak interactions between collagen and the inorganic phases [5].

Table 3. 7 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of 1.5 wt% Collagen, 1.5%Coll/MBG_SD_Sr4% and 1.5%Coll/MBG_SG_Sr4% systems.

	Storage modulus (G')	Loss modulus (G'')	Denaturation Temperature
1.5 wt% Collagen	359.7 $\pm$ 23.4 Pa	57.4 $\pm$ 9.3 Pa	41.2 $\pm$ 0.5 $^{\circ}$ C
1.5%Coll/MBG_SD_Sr4%	213.9 $\pm$ 42.8.4 Pa	36.5 $\pm$ 11.2 Pa	43.7 $\pm$ 0.6 $^{\circ}$ C
1.5%Coll/MBG_SG_Sr4%	434.9 $\pm$ 64.3 Pa	30.8 $\pm$ 15.6 Pa	45.1 $\pm$ 0.9 $^{\circ}$ C

The rheological assessment of Sr-MBG containing systems proved their potential suitability for extrusion printing applications. However, the low value of viscosity and yield stress alongside the time gap required to form a stable gel, may prevent the immediate stabilisation of the material upon deposition, leading to a limited printing fidelity [36,37]. Moreover, the poor visco-elastic properties registered for the different reconstituted collagenous matrices suggested the need of an additional chemical treatment to create intermolecular covalent bonds able to improve the final strength and stability of the printed biomaterial [17].

Based on the rheological analysis, 1.5 wt% collagen concentration was selected as the best candidate to enhance the final properties of the systems, while the influence of MBG_SD_Sr4% and MBG_SG_Sr4% particles was further investigated by means of morphological and physico-chemical studies.

3.2.2 Micro-Nano Structure Evaluation by Field Emission Scanning Electron Microscopy (FE-SEM)

The natural physical gelation of collagen is normally obtained by means of the neutralisation of acidic-solubilised solutions of collagen and the subsequent incubation at 37 $^{\circ}$ C, thus reproducing similar physiological conditions, where collagen molecules can organise and assemble into a gel matrix containing a large amount of water [16]. However, potential interactions with other materials or particular synthesis conditions can alter or prevent the natural self-assembling of collagen molecules [9]. The micro- and nanostructure of collagen/Sr-MBG composites were, thus, investigated by field emission scanning electron microscopy (FE-SEM) at different magnifications (Figure 3.9).

FE-SEM images proved the successful reconstitution of collagen fibrils forming fibrous meshes and walls that properly embedded the Sr-MBG particles and their organization at the micro and nano-scale in the fibrous matrix. Any significant agglomerate of particles was detected confirming a good dispersion of the inorganic phase inside the collagenous matrix, also suggesting that no sedimentation processes occurred during the suspension preparation [15].

Despite the use of nano-sized particles, cross-sectional FE-SEM images of Coll/MBG_SG_Sr4% showed very few aggregates not greater than 1-2 μm , confirming the good dispersion of the inorganic phase in the system.

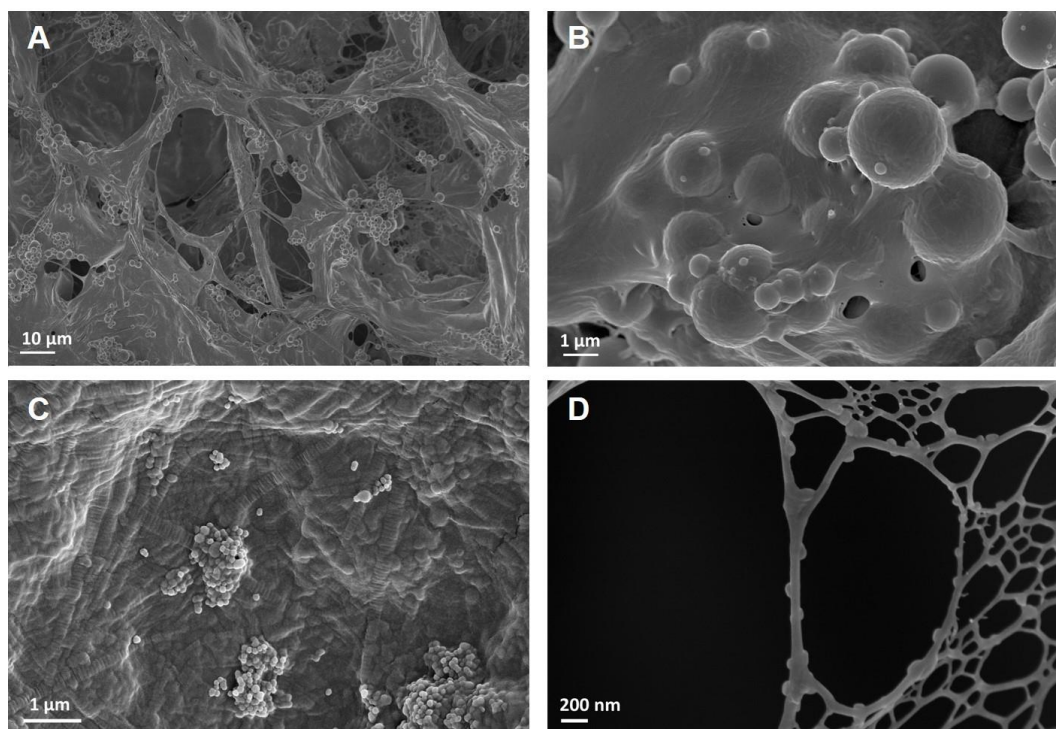


Figure 3. 9 Cross-sectional FE-SEM images of Coll/MBG_SD_Sr4% (A, B) and Coll/MBG_SG_Sr4% (C, D) samples at different magnifications.

The ability of collagen to organise in three-dimensional fibrous structures was further confirmed at higher magnifications (250000 $\times$), where the collagen fibres exhibited the typical D-periodic banding pattern ($D = 67 \text{ nm}$), due to the presence of overlapping and lacunar regions in the highly organised supramolecular packing (Figure 3.10). In addition, fibre diameters, detected from FE-SEM images, ranging from about 70 to 170 nm, were found to be consistent with data reported in the literature (50–500 nm) [15,31].

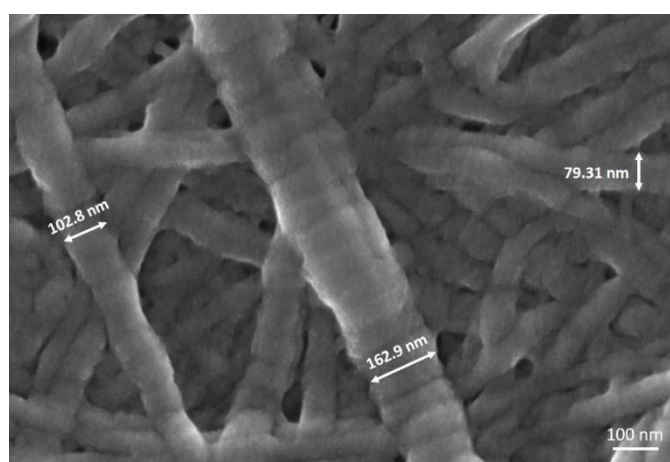


Figure 3. 10 Cross-sectional FE-SEM image of lyophilized Coll/MBG_SD_Sr4% sample at 250,000 $\times$, showing the typical banded pattern of collagen fibres.

Despite the large amount of the inorganic phase in the system composition, the natural fibrillar organisation of collagen was preserved together with the homogeneous particle embedding, both key factors for the final design of biomimetic structures.

Considering the Coll/MBG_SG_Sr4% system, in several areas of the sample cross-section (Figure 3.9D), the distribution of the MBG particles along the collagen fibrils led to the formation of nanostructures nicely resembling the dispersion of the mineral phase within the collagenous bone matrix, suggesting that the use of nano-sized MBGs can lead to more biomimetic structures.

Biomimetic nanostructured systems have proved to be beneficial not only for the osteogenic differentiation of stem cells but also to accelerate the mineralization kinetics *in vivo*. Therefore, the use of MBG_SG_Sr4% could contribute beneficially to the overall system quality [33,34].

3.2.3 *In vitro* bioactivity of Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%

One of the most important aspect in bone tissue engineering is the extracellular matrix biomineralization [6,8]. The *in vitro* bioactivity of MBGs is related to their ability to stimulate the precipitation and deposition of calcium phosphates when in contact with physiological fluids [5].

The material ability to promote the deposition of bone-like apatite minerals particularly due to the presence of MBG particles, was thus examined *in vitro* by incubating Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% samples in simulated body fluid (SBF) for up to seven days.

FE-SEM analyses revealed the formation of HA crystals homogeneously distributed on the sample sections already after three days in SBF for both systems, confirming the remarkable bioactivity of the material [7,35]. Images collected after up to seven days showed the presence of HA deposits particularly at the MBG particle surface even when significantly embedded in the fibrous collagenous matrix, as shown in Figure 3.11. With increasing immersion times in SBF, a greater amount of deposits was observed for the Coll/MBG_SD_Sr4% system, while nanoparticles of Coll/MBG_SG_Sr4% showed a surface almost completely covered by hydroxyapatite crystals already after 3 days of incubation (Figure 3.11C). These results are in agreement with the greater bioactive behaviour and the faster HA deposition kinetics observed for MBG_SG_Sr4% particles when compared to micro-sized MBGs, mainly due to their higher surface area [10,36].

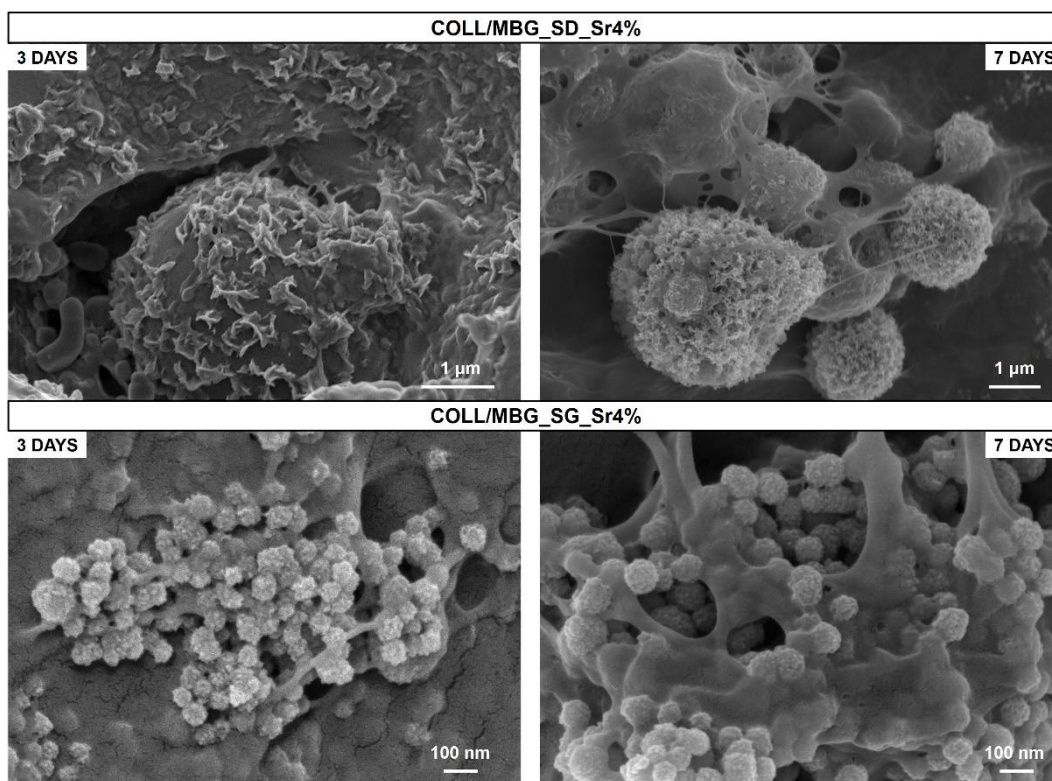


Figure 3. 11 Cross-sectional FESEM images showing HA crystal deposition on Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% samples after three and seven days of incubation in SBF at different magnifications.

Samples collected after 7 days of incubation in SBF were also analysed by means of XRD and ATR-FTIR to confirm the chemical composition and structure of the deposits.

The resulting XRD spectra reported in Figure 3.12 clearly showed the presence of crystalline peaks of HA superimposed on the broad reflection peak shown by both Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% systems, less visible after 7 days probably due to presence of the calcium phosphate layer. The detected peaks proved to match the reference values for HA reported by the International Centre for Diffraction Data [37,38]. In detail, the mineralization of Coll/MBG_SD_Sr4% samples resulted in the formation of peaks at 26°, 31.9° and 46.8° whereas the analysis on Coll/MBG_SG_Sr4% samples indicated the growth of new peaks at 31.9° and 46°. Furthermore, the narrow peak at 31.9° displayed by the Coll/MBG_SG_Sr4% diffractogram demonstrated the high crystallinity of HA [39].

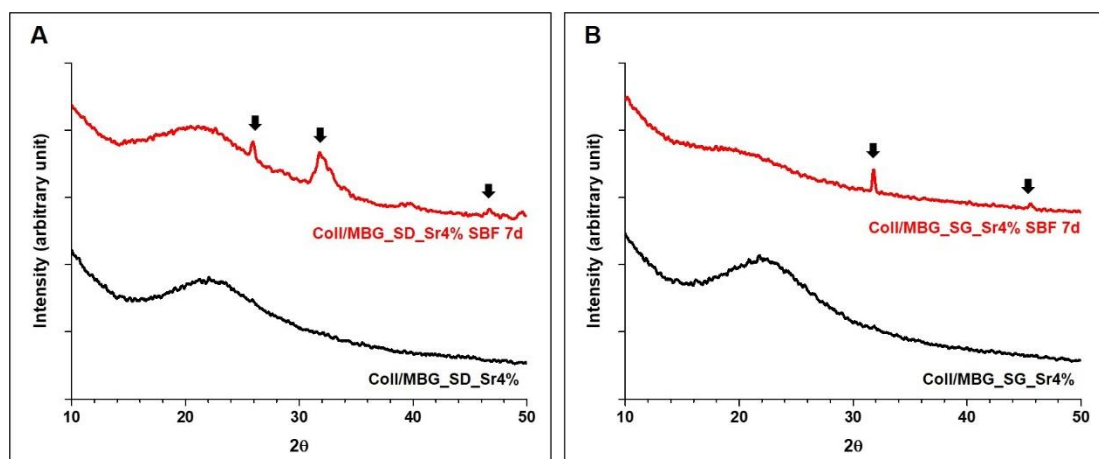


Figure 3.12 XRD patterns of Coll/MBG_SD_Sr4% (A) and Coll/MBG_SG_Sr4% (B) samples after 7 days of incubation in SBF.

ATR-FTIR analysis on samples collected after 7 days of incubation in SBF were compared with the spectra obtained from the Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% systems before incubation and collagen alone, as presented in Figure 3.13.

In the ATR-FTIR graphs the typical bands of collagen centred at about 1640 cm^{-1} , 1545 cm^{-1} , and 1236 cm^{-1} for amide I (C=O stretching), amide II (N-H stretching), and amide III (C-N bending), respectively, were observed, while the presence of MBGs was confirmed by the Si-O-Si band at 1027 cm^{-1} and 1033 cm^{-1} for Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% respectively [9,40,41].

ATR spectra of samples incubated for seven days in SBF clearly suggested the deposition of calcium phosphates on the collagenous matrices. In detail, the phosphate deposition led to a significant increase in absorbance at about 960 cm^{-1} and between 1000 and 1100 cm^{-1} , where the peak at 1053 cm^{-1} and 1073 cm^{-1} resulted from both Si-O-Si and phosphate contributions in Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% spectrum, respectively. In addition, carbonate formation was identified for both systems by the peak at about 1451 cm^{-1} [6,8]. The nature of the detected peaks was confirmed by other studies focused on bioactive glass and collagen composites after 7 or 14 days of incubation in SBF [6].

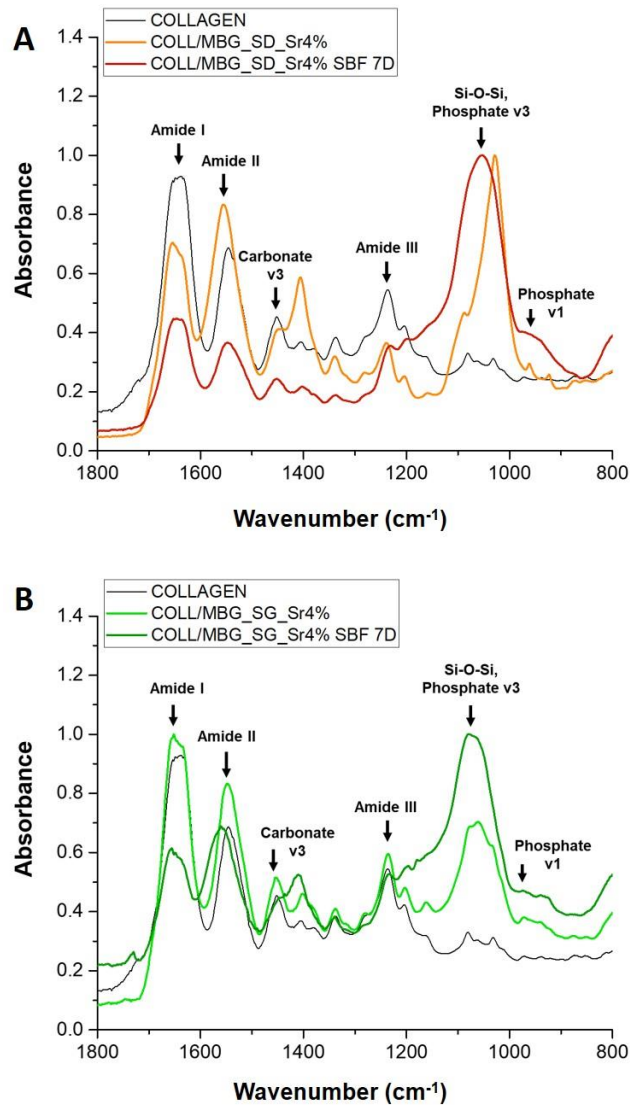


Figure 3. 13 ATR-FTIR spectra of Collagen, Coll/MBG_SD_Sr4% and Coll/MBG_SD_Sr4% after 7 days in SBF (A) and Collagen, Coll/MBG_SG_Sr4% and Coll/MBG_SG_Sr4% after 7 days in SBF (B).

Table 3.8 and 3.9 report the main peaks and the correspondent wavenumbers for each system before and after incubation in SBF.

Table 3. 8 ATR-FTIR characteristic peaks of Collagen, Coll/MBG_SD_Sr4% and Coll/MBG_SD_Sr4% after 7 days in SBF.

	Amide I (C=O stretching)	Amide II (NH stretching)	Carbonate v3	Amide III (CN bending)	Si-O-Si, Phosphate v3	Phosphate v1
Collagen	1640 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1236 cm ⁻¹	-	-
Coll/MBG _SD_Sr4%	1643 cm ⁻¹	1555 cm ⁻¹	1451 cm ⁻¹	1239 cm ⁻¹	1027 cm ⁻¹	-
Coll/MBG _SD_Sr4% SBF 7D	1645 cm ⁻¹	1548 cm ⁻¹	1451 cm ⁻¹	1232 cm ⁻¹	1053 cm ⁻¹	964 cm ⁻¹

Table 3. 9 . ATR-FTIR characteristic peaks of Collagen, Coll/MBG_SG_Sr4% and Coll/MBG_SG_Sr4% after 7 days in SBF.

	Amide I (C=O stretching)	Amide II (NH stretching)	Carbonate v3	Amide III (CN bending)	Si-O-Si, Phosphate v3	Phosphate v1
Collagen	1640 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1236 cm ⁻¹	-	-
Coll/MBG _SG_Sr4 %	1645 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1235 cm ⁻¹	1033 cm ⁻¹	-
Coll/MBG _SG_Sr4 % SBF 7D	1650 cm ⁻¹	1560 cm ⁻¹	1451 cm ⁻¹	1235 cm ⁻¹	1073 cm ⁻¹	973 cm ⁻¹

In conclusion, FESEM microscopy, XRD and ATR-FTIR analyses successfully confirmed the high bioactive behaviour of the developed hybrid systems. The significant bioactive properties can be attributable to the presence of both collagen and MBGs, as reported by other studies [5,9]. In details, the release of silicic acid due to MBG dissolution and the surface hydroxyl groups are able to induce apatite formation through ion-exchange with calcium and phosphate ions of the soaking medium. At the same time, the carboxylic groups of collagen can easily bind to calcium ions, providing nucleation sites for the further deposition of calcium phosphates without altering the triple helix structure [5,30].

3.2.4 Strontium ion release from Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%

The release of strontium ions is widely recognized to potentially enhance the pro-osteogenic properties of the materials, promoting new bone formation *in vivo* [12,42,43]. Strontium ions, thanks to its similarity with calcium, are able to activate the calcium sensing receptors on osteoblast surface, stimulating the production of osteoprotegerin (OPG) that inhibits the expression of the nuclear factor kappa beta ligand (RANKL) responsible for the osteoclastogenesis [44].

Based on these observations, the ability of MBG particles to release Sr²⁺ when embedded within the collagenous matrix was assessed and compared to data obtained for Sr-containing MBGs alone. In particular, previous studies on Sr-MBGs proved an almost complete release of the strontium content already after 3 hours of incubation in Tris-HCl both for micro- and nano-sized particles [10].

For all the performed analyses, the percentage of ion released was calculated considering the amount of strontium initially incorporated in MBG particles (as measured by ICP on acid-digested samples) and the content of inorganic phase, alone or incorporated into the hybrid formulations as described in Section 2.2.10.

During the synthesis protocol of the collagenous hybrid formulation, MBG_SG_Sr4% are suspended in 0.5 M acetic acid by sonicating and stirring at RT up to 3 hours before addition into the collagen solution, in order to disrupt any aggregate present. Therefore, the potential release of strontium ions from MBG_SG_Sr4% matrices in the 0.5 M acetic acid solution (pH 2.3) at RT was monitored and compared to that measured in a saline solution of Tris-HCl (pH 7) at different time points (3h, 10h, 24h, 3d, 7d), particularly focusing on the percentage of ion loss at 3 hours.

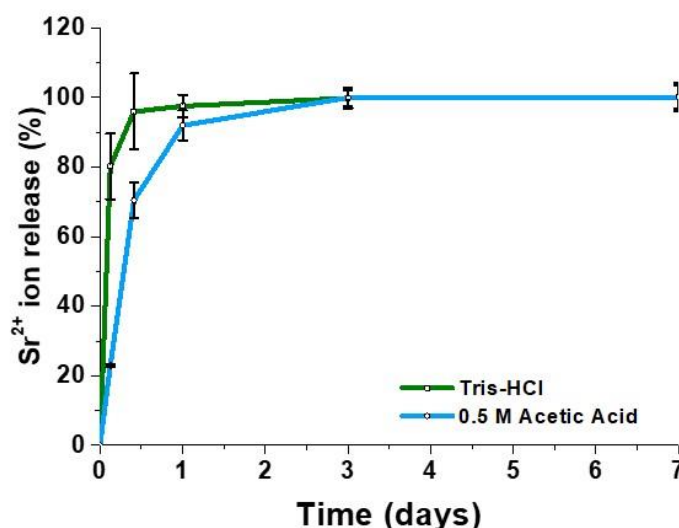


Figure 3. 14 Sr^{2+} ion release from MBG_SG_Sr4% incubated in 0.5 M acetic acid and Tris-HCl up to 7 days.

As visible from Figure 3.14, MBG_SG_Sr4% particles showed almost the total release of strontium within 24 hours of incubation in both acetic acid and Tris-HCl solutions. However, a slower release kinetic was detected when samples were incubated in 0.5 M acetic acid, registering about 22.9% of ion loss at 3 hours instead of 80.2% measured when the inorganic phase was immersed in Tris-HCl, as reported in Table 3.10. The slower release kinetics observed in acetic acid can be ascribed to the absence of ions in the solution, where the release of strontium is mainly due to diffusion mechanisms. These results proved that the time requested to create a homogeneous suspension of the nanoparticles before addition to collagen and the resulting ion release kinetics was suitable to avoid a massive loss of strontium during the material preparation process.

Table 3. 10 Sr^{2+} release values (%) from MBG_SG_Sr4% in 0.5M acetic acid and Tris-HCl at different time points (3h, 10h, 24h, 3d, 7d).

	3h	10h	24h	3d	7d
Sr^{2+} release (%) in Tris-HCl	80.2% ± 9.4	95.5.2% ± 10.9	97.5% ± 3.2	99.8% ± 2.3	100.0% ± 3.9
Sr^{2+} release (%) in acetic acid	22.9% ± 0.4	70.4% ± 5.1	91.8% ± 4.2	99.9% ± 2.9	100.0% ± 3.9

The release of Sr^{2+} ions was subsequently investigated from Sr-MBGs embedded in the collagenous matrix by soaking bulk samples in Tris-HCl at 37 °C and compared to the inorganic phases alone. Figure 3.15 showed the percentage of ion loss measured in the supernatant collected at different time points (3 h, 10h, 24 h, 3 days and 7 days) from Coll/MBG_SG_Sr4% and Coll/MBG_SD_Sr4%. in Tris-HCl for both the systems.

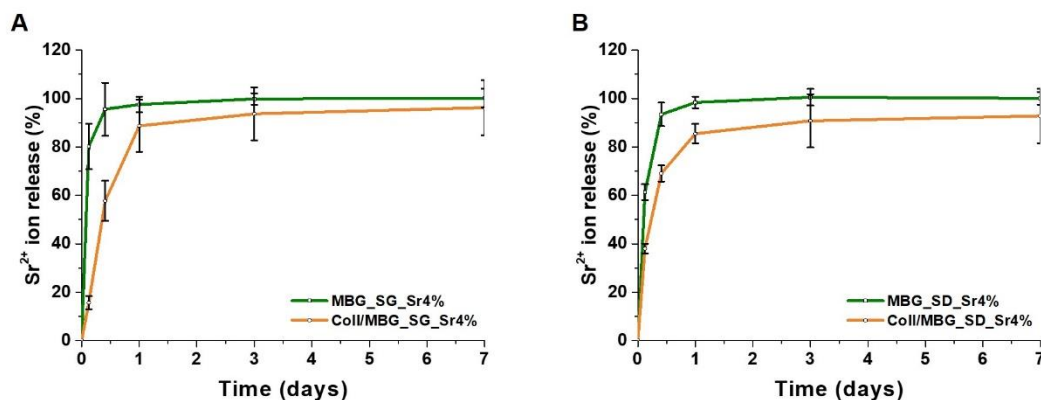


Figure 3. 15 Sr^{2+} ion release from MBG_SG_Sr4%, Coll/MBG_SG_Sr4% (A), MBG_SD_Sr4%, Coll/MBG_SD_Sr4% (B) samples incubated in Tris-HCl at 37 °C up to 7 days.

According to previous studies, both MBG_SG_Sr4% and MBG_SD_Sr4% showed an evident burst release already after 3 hours of incubation, registering 80.2% and 61.3% of ion loss as reported in Table 3.11. The faster release registered for MBG_SG_Sr4% particles can be linked to the higher surface area compared to MBG_SD_Sr4% [10].

When compared to the release data obtained from inorganic particles alone, the strontium ion burst release was delayed by about 24 h, whereas only 38.1% and 15.6% of ion loss were detected after 3 h of Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4% immersion, respectively (Figure 3.15).

The whole content of ions was released after 10 hours and 24 hours from MBG_SG_Sr4% and MBG_SD_Sr4%, as visible from Table 3.11. On the contrary, a more sustained release was registered when the inorganic phase was embedded in the polymeric matrix, according to what observed in other studies [36]. However, more than 90% of ion loss was calculated after 3 days of incubation for both Coll/MBG_SD_Sr4% and Coll/MBG_SG_Sr4%.

Table 3. 11 Sr^{2+} release values (%) from MBG_SG_Sr4%, MBG_SD_Sr4%, Coll/MBG_SG_Sr4% and Coll/MBG_SD_Sr4% in Tris-HCl at different time points (3h, 10h, 24h, 3d, 7d).

	3h	10h	24h	3d	7d
Sr^{2+} release from MBG_SG_Sr4%	80.2% $\pm$ 9.4	95.5.2% $\pm$ 10.9	97.5% $\pm$ 3.2	99.8% $\pm$ 2.3	100.0% $\pm$ 3.9
Sr^{2+} release from Coll/MBG_SG_Sr4%	15.6% $\pm$ 2.7	57.7% $\pm$ 8.2	88.7% $\pm$ 10.7	93.6% $\pm$ 10.9	96.2% $\pm$ 11.3
Sr^{2+} release from MBG_SD_Sr4%	61.3% $\pm$ 3.2	93.4% $\pm$ 4.8	98.3% $\pm$ 2.3	99.5% $\pm$ 3.4	100.0% $\pm$ 2.5
Sr^{2+} release from Coll/MBG_SD_Sr4%	38.1% $\pm$ 2.1	69.1% $\pm$ 3.3	85.4% $\pm$ 4.1	90.7% $\pm$ 10.9	92.8% $\pm$ 11.1

The resulting data suggested that the collagenous matrix did not prevent the ion exchange between the inorganic particles and the soaking medium but slowed down the release due to the medium diffusion kinetics.

Some studies on Sr-containing particles have proved the significant pro-osteogenic effect induced by Sr^{2+} species released with similar kinetics, highlighting the importance of these systems in view of bone regeneration applications [10–12]. In addition, a further control of the release kinetics could be achieved by functionalizing the external surface of the bioactive glass particles with a degradable coating or exploiting chemical crosslinking strategies for the polymeric phase [1].

In conclusion, the biomimetic organisation of nanoparticles along collagen fibrils, the high bioactive character and the higher visco-elastic properties suggested the greater potential of 1.5%Coll/MBG_SG_Sr4% as bioactive biomaterial ink for the design of biomimetic bioactive scaffolds, compared to the 1.5%Coll/MBG_SD_Sr4% system.

Based on these observations, 1.5%Coll/MBG_SG_Sr4% was selected for further investigation and used to design high resolution composite scaffolds, as described in Chapter 4.

3.3 Design and characterisation of the nano-HA containing hybrid formulations (Coll/nano-HA)

Part of the work described in this chapter has been previously published [45].

This section will describe the development and characterisation of hybrid formulations combining type I collagen and rod-like nano-sized hydroxyapatite (nano-HA), with particular focus on the proper homogenization and the physico-chemical features of the systems exploiting cytocompatible dispersing agents alongside their potential use as bioactive biomaterial inks for extrusion printing applications.

One of the most common applications of nano-HA in the biomedical field is its use as reinforcing phase within polymeric matrices to develop composite materials for bone scaffold fabrication, where type I collagen, as main organic phase of bone, is considered one of the most suitable matrices for bone regeneration applications [31]. Furthermore, collagen-nanoHA composites are highly biomimetic systems able to combine the flexibility and toughness associated with the collagenous matrix with the high compressive strength of the inorganic phase [46]. However, to develop hybrid formulations suitable for extrusion printing applications, specific rheological properties and a proper distribution of HA particles are required.

Based on the expertise of the research group, a hydrothermal method combined with the use of the cytocompatible dispersing agent Darvan 821-A was optimised to obtain uniform-sized rods with length of 40-60 nm and a width of 20 nm, as shown in Figure 3.16, thus mimicking the morphology and the size of HA crystals present in bones (50 x 25 x 2 nm) [47].

The hydrothermal treatment plays a key role in favouring the formation of HA rod-like structures, as the involved high pressure and temperature promote the growth along a preferential direction of the HA crystal nuclei [44]. Furthermore, by controlling the temperature and the duration of the hydrothermal treatment is possible to tailor the rod length [45].

The resulting particle characterisation revealed a sub-stoichiometric composition, with a Ca/P ratio equal to 1.5, close to that of the inorganic phase of bone. Moreover, the crystallite size calculated based on XRD analysis, resulted to be 38.8 nm, thus confirming the nanometric size of the nano-HA particles [45,48].

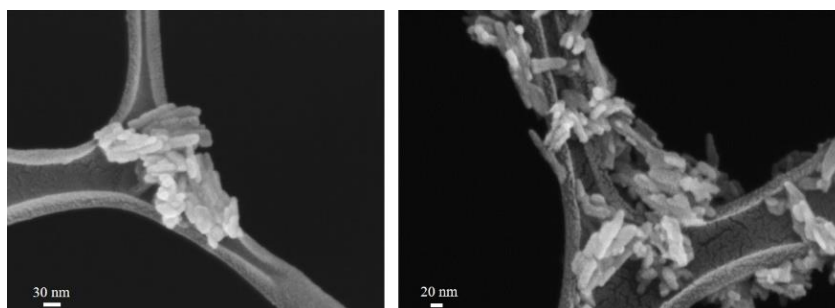


Figure 3. 16 FE-SEM images of rod-like nano-HA used to develop collagen-based hybrid formulations.

One of the main limitations in the fabrication of collagen/HA constructs is the achievement of a homogeneous distribution of HA particles throughout the embedding polymeric matrix. For this reason, the use of biocompatible polymeric dispersants such as chitosan or, in alternative, the in-situ mineralisation of collagenous scaffolds during the fabrication process have been proposed as promising approaches to avoid the HA aggregation in the resulting systems [54,55]. However, the in-situ formation of HA crystals hinders the control over the final purity and crystallinity obtained as well as the ratio between organic and inorganic phase. Furthermore, the introduction of additional polymers acting as dispersing agents in the material formulation alters the original biomimetic composition of collagen/HA systems. In addition, collagen/HA systems developed for 3D printing applications usually consider low concentration of inorganic phase in order to

promote the proper extrusion of the material, where greater needle diameters are preferred to prevent clogging during the process [21,49].

In the present work, Darvan 821-A, an anionic surfactant [51], was exploited to pre-disperse rod-like nano-HA particles before their combination with the collagen solution, without altering the final composition and volume ratio between type I collagen and inorganic particles (53% vol. of collagen and 47% vol. of inorganic phase) [17], obtaining final concentrations of 35 wt% of collagen and 65 wt% of HA [40]. The use of a dispersing agent was exploited to reduce the aggregation tendency of HA particles in aqueous-based environments, as schematically represented in Figure 3.17. In fact, HA particles are strongly inclined to aggregate due to their thermodynamic-driven tendency of reducing their entropy through the decrease of their surface energy [56]. The introduction of Darvan, which adsorbs on particle surface leading to higher negative values of zeta potential, leads to the formation of intra-particles repulsive forces, resulting in higher stable suspensions [57].

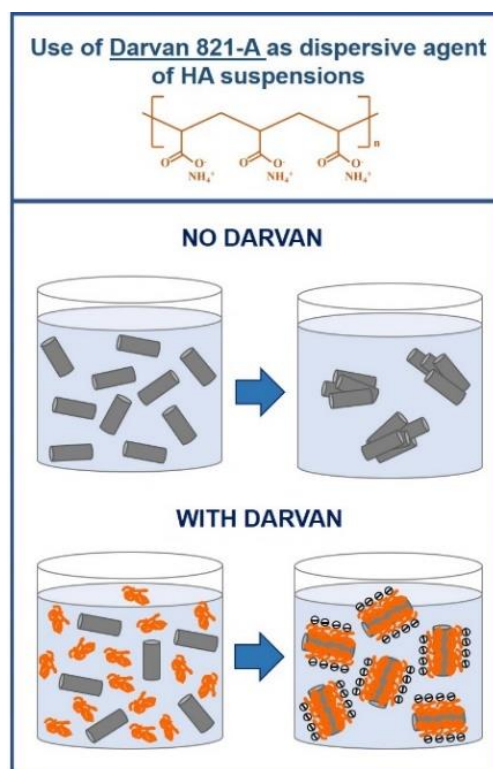


Figure 3. 17 Schematic representation of the use of Darvan 821-A as dispersive agent for HA suspensions.

Different concentrations of Darvan 821-A (0, 0.5 and 3 vol.%) were investigated in order to select the most appropriate one to obtain a homogenous system, whose rheological properties are suitable for the extrusion printing process.

To obtain the hybrid formulations, the rod-like HA nanoparticles were dispersed in Darvan 821-A basic solution (1M NaOH) by sonication for 1 hour and stirring overnight and afterwards they were incorporated into the collagen solution. Based on the analysis performed on Sr-MBG containing systems, a final concentration of about 1.5 wt% of type I collagen was selected to create the hybrid formulation. The distribution of the inorganic phase particles as well as the physico-

chemical and the rheological properties of the systems (Coll/nano-HA(0%D); Coll/nano-HA(0.5%D) ; Coll/nano-HA(3%D)) were investigated to define the most appropriate concentration of the dispersing agent.

The optimised formulation, named as Coll/nano-HA, was further studied to prove its suitability as biomaterial ink for the design of biomimetic scaffolds.

3.3.1 Morphological and physico-chemical characterisation of Coll/nano-HA

The inorganic phase dispersion and the potential successful reconstitution of the collagen fibrils were investigated by means of FE-SEM analysis. Coll/nano-HA suspensions containing different concentrations of dispersing agent were allowed to gel at 37 °C to obtain bulk samples and subsequently lyophilised to perform the morphological assessment.

As evidenced in Figure 3.18, the inorganic particles were successfully dispersed and properly embedded in the organic collagenous matrix in presence of the dispersing agent (B, C). The presence of HA nanoparticles and the use of Darvan 821-A did not hinder the reconstitution of the fibrous collagen structure naturally occurring at pH 7.4 and 37°C (physiological pH and temperature) as clearly shown in Figure 3.19.

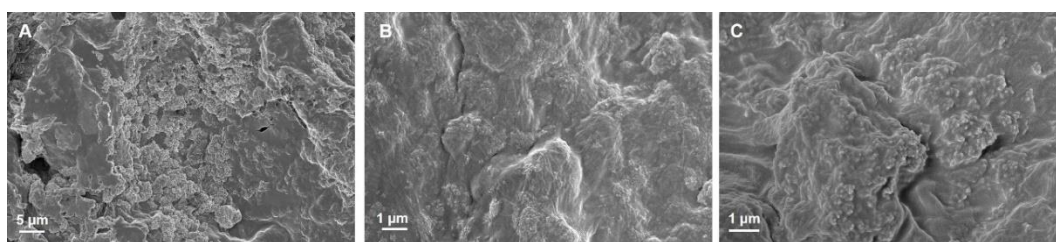


Figure 3. 18 FESEM images of Coll/nano-HA systems containing 0 vol.% (A), 0.5 vol.% (B) and 3 vol.% (C) Darvan 821-A.

No significant agglomeration was observed for nano-HA particles previously suspended in Darvan solutions, except for the presence of few particle clusters less than 1 micron in size. On the contrary, the hybrid formulation realised without the use of Darvan 821-A (Figure 3.18 A) showed the evident formation of agglomerates, where HA particles were also less embedded into the collagen matrix.

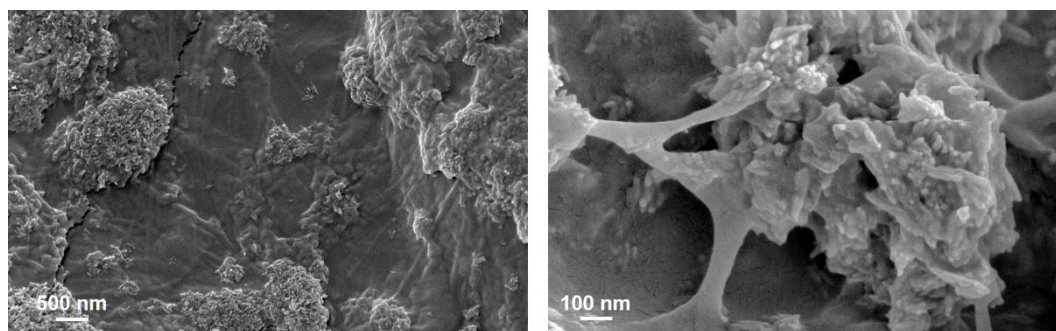


Figure 3. 19 FESEM images of Coll/nano-HA systems containing 0.5 vol.% Darvan 821-A at higher magnifications.

The morphological assessment thus confirmed the formation of homogeneous systems with a satisfactory distribution of rod-like HA particles into the collagenous phase. The previous dispersion of rod-like nano-HA in 1 M NaOH both at 0.5% and 3% v/v of Darvan 821-A proved its beneficial effects in preventing HA agglomeration, leading to the formation of nanoparticle clusters less than 1 μm in size. In addition, the presence of the anionic surfactant did not hinder the reconstitution of collagen fibrils and the formation of a final fibrous structure triggered by simil-physiological conditions. On the contrary, the absence of the dispersing agent led to the detection of big clusters of nano-HA in addition to a reconstitution of a more compact and less fibrous collagenous matrix.

To detect potential interactions between the organic and the inorganic phase, ATR-FTIR spectra of Coll/nano-HA(0%D), Coll/nano-HA(0.5%D) and Coll/nano-HA(3%D) were obtained and compared with the spectrum of collagen, as shown in Figure 3.20.

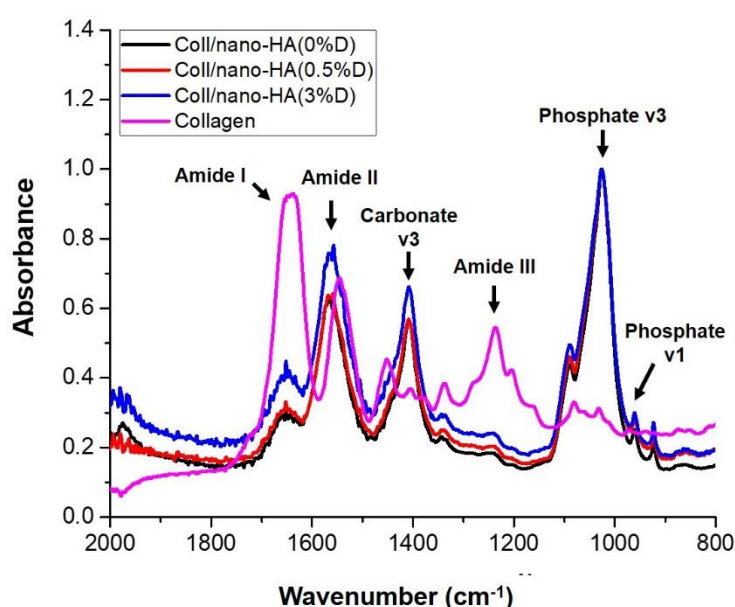


Figure 3. 20 ATR-FTIR spectra of Collagen, Coll/nano-HA(0%D), Coll/nano-HA(0.5%D) and Coll/nano-HA(3%D).

As expected, all the reported spectra reported the typical absorbances of collagen at about 1640 cm^{-1} , 1545 cm^{-1} and 1236 cm^{-1} and correspondent to amide I, amide II and amide III respectively, where the HA containing systems presented a slight shift of the peaks towards higher wavenumbers (Table 3.12). Moreover, particularly amide I and amide III bands were less evident for all the Coll/nano-HA spectra compared to the pure collagen. According to other studies in the literature [39,50–53], electrostatic interactions between opposite charged components as well as hydrogen bonding can occur between collagen and hydroxyapatite. The electrostatic interaction is particularly strong between Ca^{2+} cations from the HA, and carboxylate groups from collagen. Based on this evidence, small shifts in the absorption peaks of the amide I can be detected alongside a significant decrease in the peak of amide III, confirming the interaction with HA particles [54].

Table 3. 12 ATR-FTIR characteristic peaks of Collagen, Coll/nano-HA(0%D), Coll/nano-HA(0.5%D) and Coll/nano-HA(3%D).

	Amide I (C=O stretchin g)	Amide II (NH stretchin g)	Carbonat e v3	Amide III (CN bending)	Phosphat e v3	Phosphat e v3	Phosphat e v1
Collagen	1640 cm ⁻¹	1545 cm ⁻¹	1404 cm ⁻¹	1236 cm ⁻¹	1081 cm ⁻¹	1031 cm ⁻¹	-
Coll/nano-HA(0%D)	1649 cm ⁻¹	1563 cm ⁻¹	1407 cm ⁻¹	1240 cm ⁻¹	1088 cm ⁻¹	1025 cm ⁻¹	960 cm ⁻¹
Coll/nano-HA(0.5%D)	1649 cm ⁻¹	1563 cm ⁻¹	1407 cm ⁻¹	1240 cm ⁻¹	1088 cm ⁻¹	1025 cm ⁻¹	960 cm ⁻¹
Coll/nano-HA(3%D)	1649 cm ⁻¹	1563 cm ⁻¹	1407 cm ⁻¹	1240 cm ⁻¹	1088 cm ⁻¹	1025 cm ⁻¹	960 cm ⁻¹

The presence of HA particles was detected for all the systems by observing the peaks at about 1088 cm⁻¹, 1025 cm⁻¹ and 960 cm⁻¹ corresponded to different vibration modes of phosphate groups (P–O antisymmetric stretch, P–O symmetric stretch) and the carbonates detected at about 1400 cm⁻¹, without evident differences between the spectra [53].

3.3.2 Rheological assessment of Coll/nano-HA

The hybrid formulations containing different Darvan concentrations were further assessed in terms of rheological properties in order to investigate their potential as biomaterials for extrusion-based printing technologies.

The flow ramps reported in Figure 3.21 showed the shear thinning behaviour of Coll/nano-HA suspensions containing 0 vol.% (Figure 3.21A), 0.5 vol.% (Figure 3.21B) and 3 vol.% Darvan 821-A (Figure 3.21C) at 10°C clearly evidenced by a sharp decrease of the viscosity for increasing values of shear rates applied (10⁻² – 10³ s⁻¹).

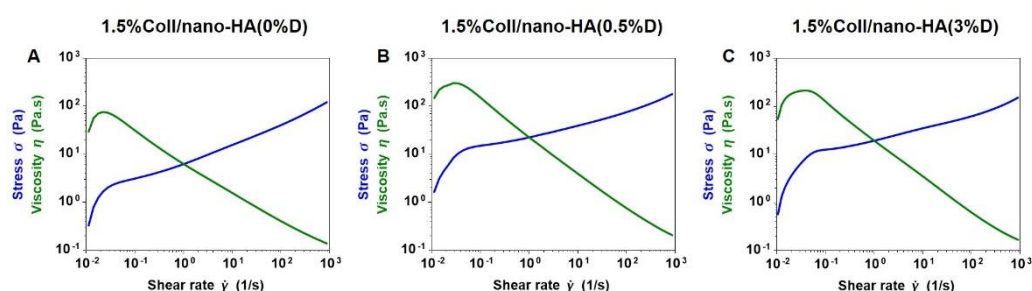


Figure 3. 21 Flow ramp analyses showing shear thinning at 10°C of the Coll/nano-HA suspension without Darvan 821-A (A) Coll/nano-HA suspension with 0.5 vol.% (B) and 3 vol.% (C) Darvan 821-A.

Compared to analogue system without the dispersing agent, Darvan-containing formulations (0.5 vol.% and 3 vol.%) led to higher values of viscosities at low shear rates, about 302.2 Pa.s and 212.1 Pa.s respectively, at variance with Coll/nano-HA system without Darvan which showed a value of about 75.1 Pa.s (Table 3.13). All

the suspensions (with and w/o Darvan) showed similar values ranging between 0.1 and 0.2 Pa.s at the highest shear rate applied. The results showed that the characteristic shear thinning behaviour of collagen was thus maintained after the introduction of nano-hydroxyapatite [23,55].

As observed for the Sr-MBG systems, the addition of the inorganic phase overall led to the increase of the material viscosity, mainly due to the interaction between collagen molecules and nano-HA particles [56]. However, an evident increase of the viscosity at low shear rates was registered only for Darvan-containing systems, probably ascribed to the higher number of interactions present between the more dispersed inorganic phases and the polymeric matrix.

Table 3. 13 Summary of viscosity values of Coll/nano-HA suspensions containing different concentrations of Darvan 821-A at 10 °C compared to 1.5% collagen solution.

	Viscosity η (max)	Viscosity η (min)
1.5 wt% Collagen	64.2 Pa.s	0.1 Pa.s
Coll/nano-HA(0%D)	75.1 Pa.s	0.2 Pa.s
Coll/nano-HA(0.5%D)	302.2 Pa.s	0.1 Pa.s
Coll/nano-HA(3%D)	212.1 Pa.s	0.1 Pa.s

The potential sol-gel transition of the systems and the gel kinetics were investigated by means of time sweep tests at 37 °C, observing the variation of G' and G'' up to 1 hour, as shown in Figure 3.22.

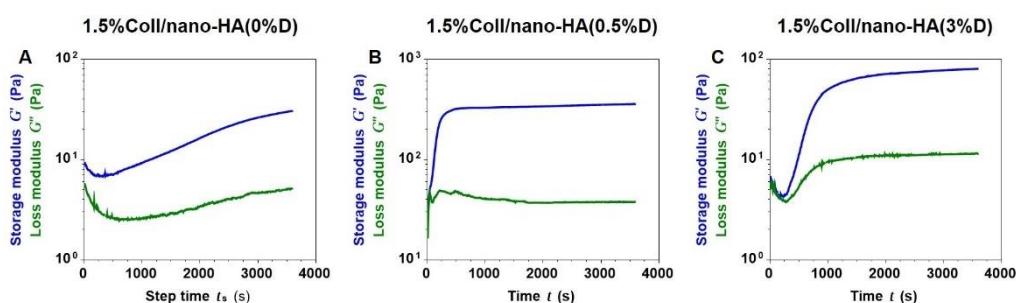


Figure 3. 22 Sol-gel transition at 37 °C of the Coll/nano-HA suspension without Darvan 821-A (A) Coll/nano-HA suspension with 0.5 vol.% (B) and 3 vol.% (C) Darvan 821-A.

The results related to time sweep tests performed applying an oscillatory stress at constant 1% strain and 1 Hz clearly showed the sol-gel transition of the materials at 37°C. However, a rapid gelation and the formation of a stable gel after about 250 seconds was obtained only for the system containing 0.5 vol.% Darvan 821-A, where a significant gap between the storage (G') and the loss (G'') modulus was observed. In addition, a slight increase of the overall visco-elastic modulus of the material was registered along the duration time of the test reaching a final value of

G' and G'' of about 358.3 Pa and 37.7 Pa for the Darvan-containing system at 0.5 vol.% and 80.3 Pa and 11.4 Pa for the formulation containing 3 vol.% Darvan, as shown in Figure 3.22 (B, C). On the contrary, the hybrid formulation without Darvan 821-A showed a poor reconstitution of the gel at 37°C, measuring 30.7 Pa and 5.2 Pa of storage and loss modulus respectively (Table 3.14).

These results suggested that the absence as well as higher concentrations of the dispersing agent can partially prevent the reconstitution of stable collagenous matrices, whereas a concentration of 0.5 vol.% gave the best results both in terms of viscosity at 10 °C and visco-elastic properties at 37 °C.

Table 3. 14 Summary of storage modulus (G'), loss modulus (G'') and gel point registered for Coll/nano-HA suspensions containing different concentrations of Darvan 821-A at 37 °C.

	Storage modulus (G') after 1 hour at 37 °C	Loss modulus (G'') after 1 hour at 37 °C	Gel point
Coll/nano-HA(0%D)	30.7 Pa	5.2 Pa	< 1000 s
Coll/nano-HA(0.5%D)	358.3 Pa	37.7 Pa	< 50 s
Coll/nano-HA(3%D)	80.3 Pa	11.4 Pa	< 400 s

The reported curves showed how lower concentrations of Darvan 821-A seemed to promote the reconstitution of a stronger matrix indicated by greater values of G' . These results may suggest a strong interaction of the dispersing agent not only with the inorganic phase but also with the collagen molecules, partially hindering the fibril self-assembly for increasing concentrations. At the same time, the formation of significant HA agglomerates in absence of Darvan 821-A led to the poor sol-gel transition of the hybrid system, identified by lower and similar values of storage (G') and loss (G'') moduli.

Moreover, a rapid and marked sol-gel transition at 37°C was observed only for the system containing 0.5 vol.% of Darvan, while Coll/nano-HA(0%D) and Coll/nano-HA(3%D) showed longer gelation kinetics.

The higher value of viscosity detected in case of the Coll/nano-HA system at 0.5 vol.% Darvan 821-A further suggested that lower concentrations of the dispersing agent promoted the development of systems with rheological properties able to better support the printing process.

Based on these observation, Coll/nano-HA containing 0.5 vol.% Darvan 821-A were selected to perform further analysis, enabling the comprehensive characterisation of the hybrid formulation as potential biomaterial ink. Coll/nano-HA containing 0.5 vol.% Darvan 821-A will be hereafter referred as Coll/nano-HA.

The printability of Coll/nano-HA suspension was further assessed observing the yield stress value as well as the shear recovery by means of amplitude sweep and peak hold tests at 10 °C simulating the stress conditions occurring during the printing process.

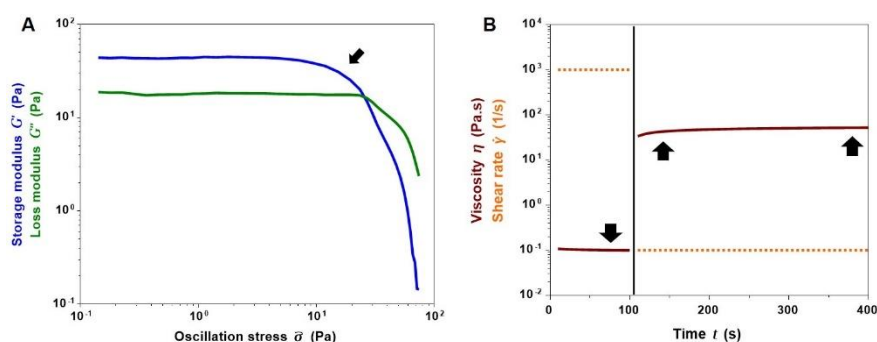


Figure 3.23 Yield stress (A) and shear recovery (B) of Coll/nano-HA suspension at 10 °C.

As shown in Figure 3.23A, Coll/nano-HA suspension showed a low yield stress of about 5.7 Pa, where the value was defined by observing the gradual decrease of G' at increasing shear stresses applied. In fact, under the yield point value, the suspension showed a solid-like behaviour proved by the higher contribution of the storage modulus G' compared to the loss modulus G'' . When shear stresses higher than 5.7 Pa were applied, the material started to flow, leading to an overall increase of the viscous contribution described by G'' . This test can be used alternatively to the flow ramp (presented in Figure 3.3 and 3.4) to define the yield point value of the material, as function of G' and G'' .

Figure 3.23B showed the shear recovery of the Coll/nano-HA suspension, proving an evident time dependence (thixotropy) as already observed for Coll/Sr-MBGs hybrid formulations. In detail, as reported in Table 3.15 the viscosity of the Coll/nano-HA suspension was gradually recovered once the material was exposed to almost zero-shear conditions, showing values of about 33.8 Pa.s immediately after removing the high shear rate and 51.3 Pa.s after 300 seconds. However, considering a viscosity of about 55.6 Pa.s at 0.1 s^{-1} , as registered during the flow ramp, the Coll/nano-HA suspension proved faster shear recovery kinetics compared to the Sr-MBG containing systems.

Table 3. 15 Yield stress and shear recovery of Coll/nano-HA compared to the values obtained for 1.5%Coll/MBG_SG_Sr4%.

	Viscosity η (1000 s ⁻¹)	Viscosity η (0.1 s ⁻¹ - 0 s)	Viscosity η (0.1 s ⁻¹ - 300 s)	Viscosity η at 0.1 s ⁻¹ (flow ramp)	Yield stress
Coll/nano-HA	0.1 Pa.s	33.8 Pa.s	51.3 Pa.s	55.6 Pa.s	5.7 Pa
1.5%Coll/MBG_SG_Sr4%	0.1 Pa.s	18.5 Pa.s	29.8 Pa.s	56.0 Pa.s	4.1 Pa

The visco-elastic properties of the composite material were further explored in order to study its strength and thermal stability after incubation at 37 °C for 3 hours. In details, the value of storage (G') and loss (G'') moduli was detected by means of a dynamic amplitude sweep analysis (0.01-1% strain) carried out at 37 °C and 1 Hz, as shown in Figure 3.24A. The same samples underwent an oscillation temperature ramp (20 °C – 60 °C) under 1% strain and 1 Hz using a ramp rate of 5 °C/min in order to observe the denaturation temperature of the system associated to the sharp decrease of the visco-elastic properties (Figure 3.24B).

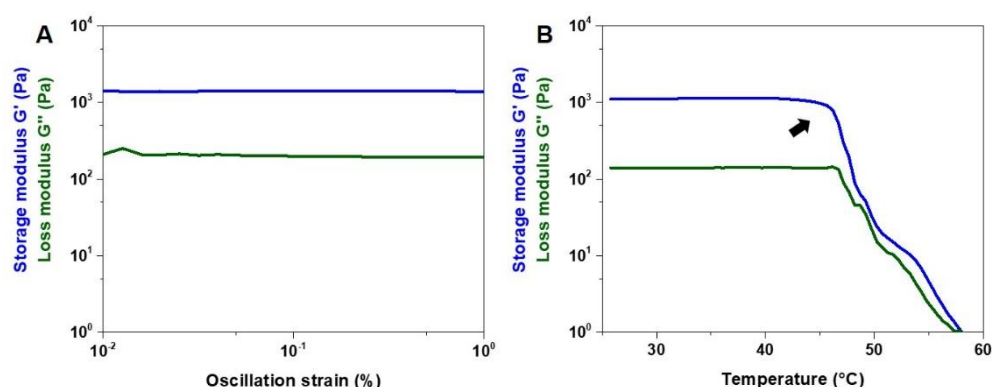


Figure 3. 24 Amplitude sweep (A) and temperature ramp (B) tests showing G' (storage modulus), G'' (loss modulus) values and denaturation temperature of Coll/nano-HA after 3 h of incubation at 37 °C.

As reported in Table 3.16, the average values of storage modulus (G') and loss modulus (G'') in the selected range of strains were measured to be about 1316 Pa and 205 Pa, respectively. The sharp increase of the overall material strength after 3 hours of incubation at 37 °C again proved that longer incubation times at 37 °C favour the self-assembling of the collagen-based systems as already shown by Coll/MBG_SG_Sr4% [10].

Coll/nano-HA samples subjected to a temperature ramp performed between 20 and 60 °C (Figure 3.24B) reported a sharp decrease of both storage and loss modulus at about 45 °C, mainly related to the denaturation of collagen and the subsequent loss of the structural and mechanical stability of the material.

Table 3. 16 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of Coll/nano-HA system.

	Storage modulus (G')	Loss modulus (G'')	Denaturation Temperature
Coll/nano-HA	1316.1 $\pm$ 56.9 Pa	205.2 $\pm$ 21.3 Pa	45 $\pm$ 0.4 °C

As previously observed for the MBG-containing systems, the reported results suggested that the further use of chemical and/or physical crosslinking methods promoting the formation of covalent bonds between collagen molecules could improve the overall mechanical and thermal stability of the material and thus that of the final scaffolds. However, the Coll/nano-HA system demonstrated enhanced rheological properties compared to the Coll/MBG_SG_Sr4% system, indicating a higher interaction between the organic and inorganic phase in the nano-HA containing composite.

3.4 Design and characterisation of the hybrid formulation containing nano-sized Sr-MBG and nano-HA particles (Coll/MBG_SG_Sr4%/nano-HA)

Based on the results obtained by the previous investigation, this section will describe the development and characterisation of a collagen-based hybrid formulation containing two different inorganic phases: MBG_SG_Sr4% and nano-HA. The goal was to combine the peculiar properties of Sr-MBG and nano-HA, obtaining a bioactive and biomimetic system potentially suitable for the extrusion printing of bone scaffolds able to release osteogenic ions and resembling the natural composition of bone tissue.

The suspension was realised considering 45 wt% of collagen, 40 wt% of hydroxyapatite and 15 wt% of MBG_SG_Sr4%, previously calculated from the volume ratio between organic and inorganic phases in the natural bone (53% vol. of collagen and 47% vol. of inorganic phase) and considering a volume ratio of 50:50 between nano-HA and MBG_SG_Sr4% [17].

MBG_SG_Sr4% inorganic phase was selected instead of micro-sized Sr-MBGs following the superior rheological properties and in order to obtain a more biomimetic nanocomposite, as demonstrated in Section 3.2. At once, a concentration of 0.5 vol% of Darvan 821-A was selected to properly disperse the nano-HA particles based on the results obtained and described in Section 3.3.

The protocols developed for Coll/MBG_SG_Sr4% and Coll/nano-HA systems were exploited and combined to optimise the preparation procedure of Coll/MBG_SG_Sr4%/nano-HA. The effect of the combination of MBG_SG_Sr4% and nano-HA was studied in terms of physico-chemical, bioactive and rheological properties, focusing on the potential of the developed hybrid formulation as biomaterial ink.

3.4.1 Morphological and physico-chemical characterisation of Coll/MBG_SG_Sr4%/nano-HA

The morphological assessment on sample cross-sections by means of FE-SEM analysis was performed to investigate the proper reconstitution of the collagen fibrils as well as the distribution of the inorganic phases into the organic matrix.

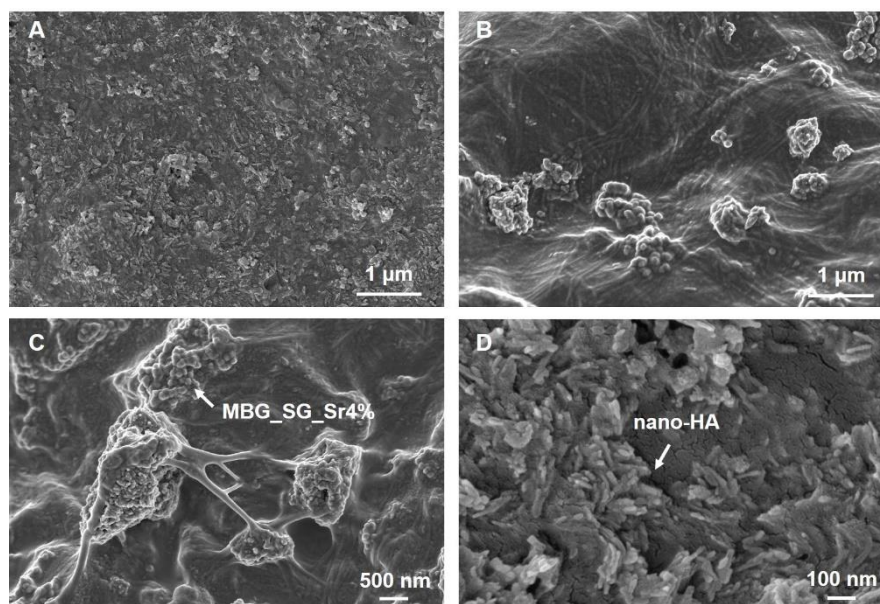


Figure 3. 25 Cross-sectional FE-SEM images of Coll/MBG_SG_Sr4%/nano-HA samples at different magnifications. The white arrows indicate the different inorganic phases embedded into the organic matrix.

FE-SEM images, reported in Figure 3.25, showed the reconstitution of the collagenous matrix as well as the good distribution and embedding of the inorganic phases. The images confirmed the presence of both nano-sized spherical MBG particles and the rod-like HA. Some agglomerates of inorganic phases were observed and measured to be lower than 5 microns in size, overall showing the formation of a homogeneous system. Images at higher magnification enabled to appreciate the proper separation of rod-like nano-HA particles and the large presence of collagen fibrils embedding some particle agglomerates.

The chemical features of the composite were further assessed by ATR-FTIR analysis as shown in Figure 3.26 and the different bands are summarised in Table 3.17.

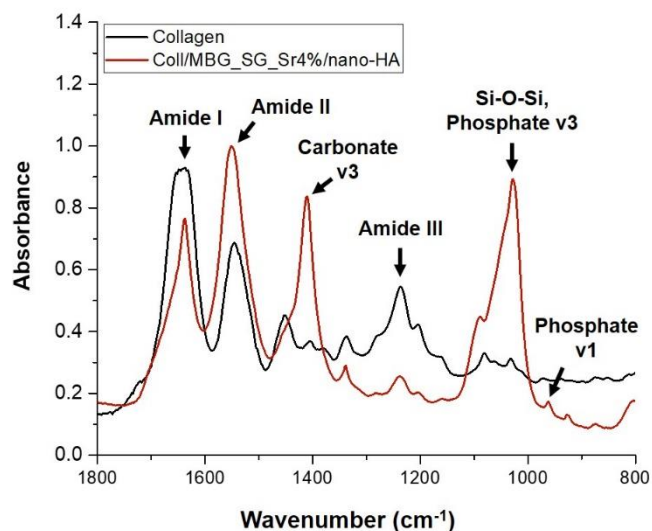


Figure 3. 26 ATR-FTIR spectra of Collagen and Coll/MBG_SG_Sr4%/nano-HA.

The ATR_FTIR spectrum of the Coll/MBG_SG_Sr4%/nano-HA system clearly presented the typical peaks of collagen centred at 1637 cm^{-1} for amide I (C=O stretching), at 1551 cm^{-1} for amide II (N-H stretching) and at 1238 cm^{-1} for amide III (C-N bending) [31,50]. As already observed for the Coll/nano-HA system, amide I and amide III bands presented a weaker contribution compared to collagen spectrum and this aspect can be ascribed to the presence of HA and the potential interactions with the collagen molecules [50,51,53].

The presence of HA was confirmed by the phosphate peaks centred at 1089 cm^{-1} and 963 cm^{-1} , while the peak at 1027 cm^{-1} resulted from the contribution of both Si-O-Si groups of MBG_SG_Sr4% and the phosphate of nano-HA [5,53,57].

No evident shifts were detected comparing the different spectra, excluding the potential formation of chemical covalent bonds between the organic and inorganic phases, as reported for the other developed systems and confirmed by different studies in the literature [5,53,56].

Table 3. 17 ATR-FTIR characteristic peaks of Collagen and Coll/MBG_SG_Sr4%/nano-HA.

	Amide I (C=O stretchi ng)	Amide II (NH stretchi ng)	Carbon ate v3	Amide III (CN bendin g)	Phosph ate v3	Si-O- Si, Phosph ate v3	Phosph ate v1
Collagen	1640 cm^{-1}	1545 cm^{-1}	1404 cm^{-1}	1236 cm^{-1}	1081 cm^{-1}	1031 cm^{-1}	-
Coll/MBG_SG_Sr4% /nano-HA	1637 cm^{-1}	1551 cm^{-1}	1411 cm^{-1}	1238 cm^{-1}	1089 cm^{-1}	1027 cm^{-1}	963 cm^{-1}

3.4.2 Bioactivity and Sr^{2+} release ability of Coll/MBG_SG_Sr4%/nano-HA

As previously studied for the Sr-MBG containing system, the potential bioactive feature of the developed hybrid formulation was investigated incubating material samples (prepared as described in Section 2.2.4) in simulated body fluid (SBF) at 37 °C up to 7 days.

FE-SEM analysis was subsequently conducted on the collected samples to detect the potential deposition of HA crystals thanks to the ionic exchange between the inorganic phase and the surrounding medium [5,6,10].

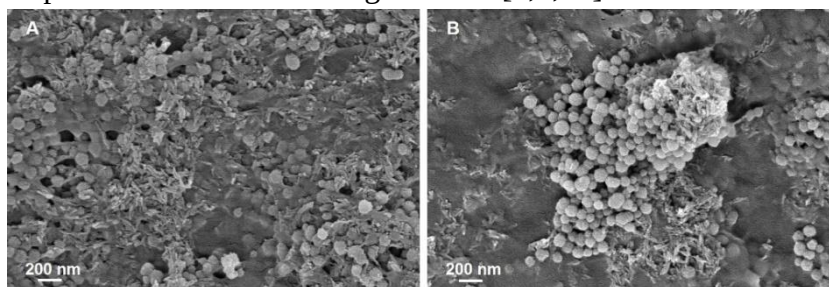


Figure 3. 27 FE-SEM images showing HA crystal deposition on Coll/MBG_SG_Sr4%/nano-HA samples after 7 days of incubation in SBF at different magnifications.

Figure 3.27 shows the morphological assessment performed on samples collected after 7 days of immersion in SBF by means of FE-SEM analysis.

Compared to the images reported in Figure 3.25, MBG_SG_Sr4% particles showed a rougher surface probably due to the presence of calcium phosphate deposits. In this case, XRD analysis on samples before and after the incubation in SBF was not considered for the bioactivity assessment, because of the presence of nano-HA particles in the material formulation, that hindered the proper detection of the deposited calcium phosphate crystals.

Alongside the bioactive properties mainly induced by the presence of MBG_SG_Sr4%, the release of Sr^{2+} from the composite material was explored by means of ICP analysis.

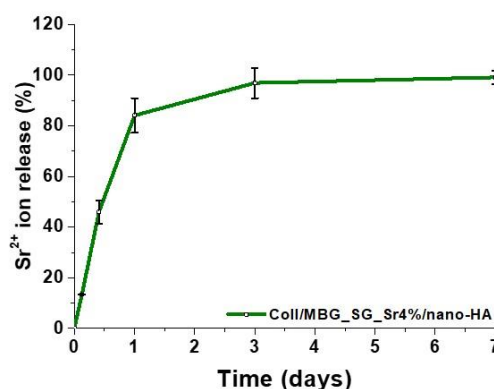


Figure 3. 28 Sr^{2+} ion release from Coll/MBG_SG_Sr4%/nano-HA incubated in Tris-HCl up to 7 days.

As shown in Figure 3.28, the release kinetics of the Coll/MBG_SG_Sr4%/nano-HA system were found to be similar to those reported by Coll/MBG_SG_Sr4%. In particular, about 84.1% of the total amount of incorporated strontium (as measured by ICP on acid-digested samples) was released after 24 hours of immersion in Tris-HCl buffer (pH 7) at 37 °C, while the complete release was obtained after 3 days (Table 3.18). However, as discussed in Section 3.2.4, the embedding of MBG_SG_Sr4% particles into the organic phase partially delayed the total loss of strontium ions normally observed after 3 hours when considering the inorganic particles alone [10,36].

Table 3. 18 Sr²⁺ release values (%) from Coll/MBG_SG_Sr4%/nano-HA in Tris-HCl at different time points (3h, 10h, 24h, 3d, 7d) compared to the results previously obtained for MBG_SG_Sr4% and Coll/MBG_SG_Sr4%.

	3h	10h	24h	3d	7d
Sr ²⁺ release Coll/MBG_SG_Sr4%/nano- HA	13.3% ± 0.1	46.1% ± 4.5	84.1% ± 6.7	96.9% ± 5.9	99.1% ± 2.5
Sr ²⁺ release from MBG_SG_Sr4%	80.2% ± 9.4	95.5.2% ± 10.9	97.5% ± 3.2	99.8% ± 2.3	100.0% ± 3.9
Sr ²⁺ release from Coll/MBG_SG_Sr4%	15.6% ± 2.7	57.7% ± 8.2	88.7% ± 10.7	93.6% ± 10.9	96.2% ± 11.3

The reported data thus proved that, alongside the biomimetic composition due to the combination of type I collagen and nano-HA, the presence of MBG_SG_Sr4% further enhanced the physico-chemical features of the composite material, overall increasing its bioactivity and potential osteogenic properties.

3.4.3 Rheology of Coll/MBG_SG_Sr4%/nano-HA

The rheological features of the hybrid formulation were studied in order to assess its potential printability and the visco-elastic properties upon the reconstitution of the collagenous matrix in simil-physiological conditions.

The variation of the material viscosity and the potential presence of a yield stress were explored by means of flow ramps at 10 °C as following the investigation used for the other hybrid formulations.

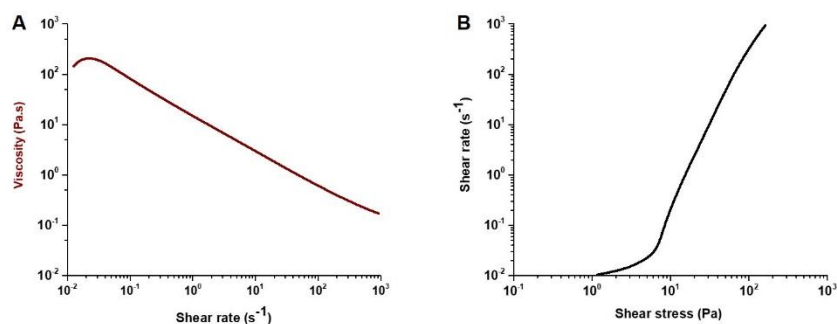


Figure 3. 29 . Flow ramps performed on Coll/MBG_SG_Sr4%/nano-HA suspension at 10 °C showing viscosity variation with increasing shear rate values (A) and the shear rate pattern as a function of stress variation (B).

Figure 3.29A showed as Coll/MBG_SG_Sr4%/nano-HA suspension kept at 10 °C was characterised by a pseudo-plastic behaviour when subjected to increasing values of shear rate ($0.01 - 1000 \text{ s}^{-1}$), similarly to what observed for Coll/Sr-MBGs and Coll/nano-HA suspensions. As summarised in Table 3.19, the suspension viscosity decreased from about 206.7 Pa.s to 0.17 Pa.s with increasing shear rates. As previously discussed, this aspect is particularly favourable in the case of materials intended for 3D printing applications, since it allows the correct extrusion especially using thin needles involving high shear rates and stresses during the process.

The measured viscosity and the yield stress of about 5.9 Pa were consistent with the values registered for the collagen formulations containing only MBG_SG_Sr4% or nano-HA particles. As previously observed, the addition of the inorganic phases did not hinder the shear thinning behaviour of collagen. However, low values of viscosity and yield stress were ascribed to the absence of strong chemical bonds between the organic and inorganic phase [5,51].

Table 3. 19 Summary of viscosity and yield stress values of Coll/MBG_SG_Sr4%/nano-HA suspension at 10 °C compared to the values obtained for Coll/MBG_SG_Sr4% and Coll/nano-HA.

	Viscosity η (max)	Viscosity η (min)	Yield stress
Coll/MBG_SG_Sr4%/nano-HA	206.7 Pa.s	0.17 Pa.s	5.9 Pa
1.5%Coll/MBG_SG_Sr4%	97.3 Pa.s	0.1 Pa.s	4.1 Pa
Coll/nano-HA	302.2 Pa.s	0.1 Pa.s	5.7 Pa

The yield point of Coll/MBG_SG_Sr4%/nano-HA was confirmed by means of the amplitude sweep test performed at 10 °C represented in Figure 3.30A. In this case, the yield stress of about 5.9 Pa was defined observing the decrease of the storage modulus (G') and the subsequent increase of the loss modulus (G'') contribution, indicative of the material flow.

The suspension shear recovery was investigated observing the variation of the material viscosity subjected to zero-shear conditions immediately after the removal of a high shear rate applied of 1000 s^{-1} , as visible in Figure 3.30B.

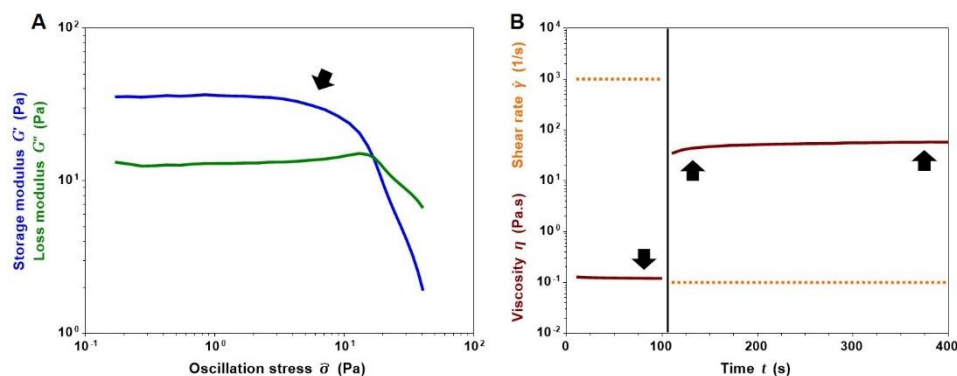


Figure 3. 30 Amplitude sweep showing yield stress (A) and printing process simulation by means of the peak hold test performed at 10°C (B) on the Coll/MBG_SG_Sr4%/nano-HA system.

Table 3.20 summarises the viscosity values of Coll/MBG_SG_Sr4%/nano-HA at different shear conditions, comparing the results obtained for the collagen solution, Coll/MBG_SG_Sr4% and Coll/nano-HA systems. The slow shear recovery indicated by the increasing values of viscosity detected after 300 seconds from the shear stress removal was already observed for collagen solutions. The results thus suggested that the addition of the inorganic phases did not affect the time requested to the system to recover the original structure and thus the viscosity values at zero-shear conditions.

Table 3. 20 Viscosity values of Coll/MBG_SG_Sr4%/nano-HA at different shear rates compared to the values obtained for 1.5%Collagen, Coll/MBG_SG_Sr4% and Coll/nano-HA.

	Viscosity η (1000 s^{-1})	Viscosity η (0.1 s^{-1} - 0 s)	Viscosity η (0.1 s^{-1} - 300 s)	Viscosity η at 0.1 s^{-1} (flow ramp)
1.5 wt% Collagen	0.06 Pa.s	5.3 Pa.s	9.5 Pa.s	19.5 Pa.s
Coll/MBG_SG_Sr4%/nano-HA	0.15 Pa.s	34.5 Pa.s	57.5 Pa.s	80.9 Pa.s
Coll/nano-HA	0.1 Pa.s	33.8 Pa.s	51.3 Pa.s	55.6 Pa.s
1.5%Coll/MBG_SG_Sr4%	0.1 Pa.s	18.5 Pa.s	29.8 Pa.s	56.0 Pa.s

The sol-gel transition of the material, triggered by the ordered packing of collagen molecules forming 3D fibril structures, was investigated by means of a time sweep test, where the suspension was loaded at 10°C and immediately subjected at a constant temperature of 37°C and an oscillatory stress (1% strain, 1 Hz) up to 1 hour.

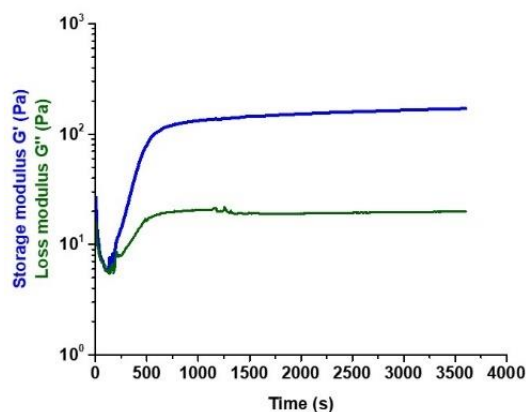


Figure 3. 31 Sol-gel transition of Coll/MBG_SG_Sr4%/nano-HA detected through the time sweep test at 37 °C.

As illustrated in Figure 3.31, the Coll/MBG_SG_Sr4%/nano-HA system presented a sol-gel transition detected as the evident increase of the storage modulus (G') compared to the loss modulus (G''), leading to the conclusion that the inorganic phase addition did not hinder the natural self-assembly of collagen.

After 1 hour at 37 °C, the storage modulus of the system visibly increased denoting the creation of a more stable and solid gel, with values of 170.3 Pa and 19.9 Pa for G' and G'' values, respectively (Table 3.21).

Compared to the systems containing only one inorganic phase, Coll/MBG_SG_Sr4%/nano-HA showed a slightly longer gel kinetics, potentially ascribed to the partial influence of the interactions between inorganic and organic phases over the self-assembly of collagen molecules. However, a stable gel was observed after 500 seconds of exposure at 37 °C, confirming the successful reconstitution of the polymeric solid matrix.

Table 3. 21 Summary of storage modulus (G'), loss modulus (G'') and gel point registered for Coll/MBG_SG_Sr4%/nano-HA system at 37 °C.

	Storage modulus (G') after 1 hour at 37 °C	Loss modulus (G'') after 1 hour at 37 °C	Gel point
Coll/MBG_SG_Sr4%/nano-HA	170.3 Pa	19.9 Pa	< 500 s

The potential increase in material strength upon longer incubation times was further assessed by means of an amplitude sweep test at 37 °C, while a temperature ramp was exploited to observe the denaturation of the composite, as represented in Figure 3.32.

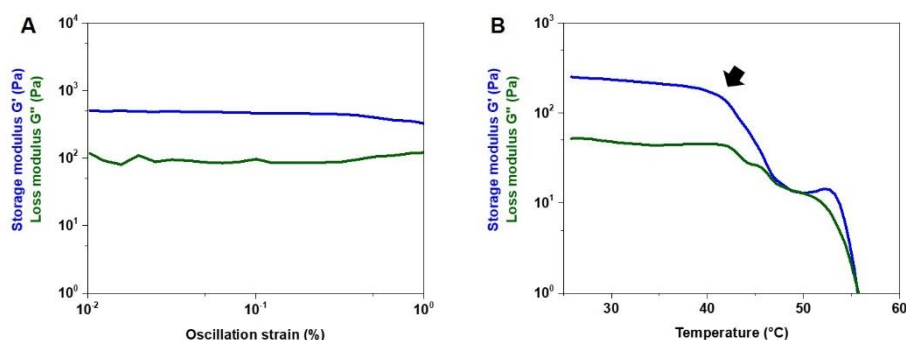


Figure 3.32 Amplitude sweep (A) and temperature ramp (B) tests showing G' (storage modulus), G'' (loss modulus) values and denaturation temperature of Coll/MBG_SG_Sr4%/nano-HA after 3 h of incubation at 37 °C.

The results presented in Figure 3.32A and summarised in Table 3.22 proved that longer incubation of the system at 37 °C promoted the molecular fibrillogenesis thus enhancing the packing of fibrils, consequently attaining higher values of material stiffness. In fact, the storage modulus G' increased from about 170 Pa to 369.5 Pa after 3 hours of exposure at 37 °C.

At the same time, the temperature ramp was performed to investigate the thermal stability of the system subjected to increased temperatures (up to 60 °C) and the subsequent variation in the viscoelastic properties. The clear drop in G' and G'' values registered at about 42 °C was related to the denaturation of collagen and the overall collapse of the structure.

Table 3.22 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of Coll/MBG_SG_Sr4%/nano-HA system.

	Storage modulus (G')	Loss modulus (G'')	Denaturation Temperature
Coll/MBG_SG_Sr4%/nano-HA	369.5 ± 31.4 Pa	91.6 ± 12.7 Pa	42 ± 0.9 °C

The rheological assessment of the Coll/MBG_SG_Sr4%/nano-HA system proved its suitability as biomaterial ink, particularly due to the observed shear thinning behaviour. However, as previously discussed for the Coll/MBG_SG_Sr4% and Coll/nano-HA systems, the low values of viscosity and yield stress, as well as the low shear recovery suggested the optimisation of strategies able to guarantee the proper support of the material after extrusion and good results in terms of printing fidelity, as will be discussed in Chapter 4. Moreover, the low stiffness values showed by the reconstituted composite matrix at 37 °C, required the further stabilisation of the material by means of cytocompatible chemical crosslinking methods.

3.5 Chemical crosslinking of the collagen-based formulations

Part of the work described in this chapter has been previously published [3,4].

The collagen-based formulations developed and described in the previous sections proved to successfully reconstitute into stable composite matrices thanks to the physical crosslinking of collagen molecules at physiological pH and temperature. However, as demonstrated by the rheological assessment of all the systems after gelation at 37 °C, the registered low values of visco-elastic moduli as well as the denaturation temperatures, suggested the need of crosslinking methods to further improve the stability.

According to different studies in the literature, the combination with inorganic phases is known to provide enhanced mechanical stiffness and stability to collagen-based matrices, but, by itself, it may be insufficient [32]. In the natural extracellular matrix, collagen molecules are covalently bonded through the action of specific enzymes, ensuring the formation of a stable and stiff matrix composed by a dense fibrillar packing [14,58,59]. Therefore, to provide the final constructs with improved mechanical features, the formation of covalent bonds between protein molecules was explored by using exogenous crosslinking methods [32]. However, some of the most used and efficient collagen crosslinkers, such as formaldehyde and glutaraldehyde, are associated with significant drawbacks such as cytotoxicity and foreign body response. Based on this observations, (1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) combined with N-hydroxysuccinimide (NHS), 4-arm polyethylene glycol succinimidyl glutarate (4-StarPEG) and genipin were selected as cytocompatible chemical crosslinkers of collagen and their effects on the properties of the composites were explored, ensuring the final biocompatibility of the system [60].

In detail, the selected chemical treatments were firstly tested on Coll/MBG_SG_Sr4% since it represented the first optimised hybrid formulation during the PhD studies. Subsequently, considering that genipin gave the best results in terms of crosslinking efficiency, its effects on Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA systems were explored.

The capability of strengthening collagen-based constructs of the different chemical agents was studied by observing the physico-chemical properties of the composites, their hydrolytic and enzymatic degradation as well as the potential increase of their visco-elastic properties. In the case of the MBG_SG_Sr4% containing systems, special attention was given to the potential loss of strontium ions already during the crosslinking treatment.

All the chemical treatments were performed incubating the material samples in crosslinking baths with the aim to optimise crosslinking procedures suitable for the further stabilisation of the 3D printed scaffolds.

3.5.1 Chemical crosslinking by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) and N-hydroxysuccinimide (NHS)

The chemistry of (1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) combined with N-hydroxysuccinimide (NHS) was exploited to induce the chemical crosslinking of collagen molecules in the Coll/MBG_SG_Sr4% system.

Carbodiimides are zero-length crosslinkers able to directly bind collagen molecules forming both inter and intra-molecular bonds. In detail, different studies report that EDC/NHS crosslinking can induce the formation of an amide bond by activating the side chain carboxylic acid groups of specific amino acids (such as aspartic and glutamic acid residues) on collagen. The subsequent aminolysis of the o-isoacylurea intermediates by the ϵ -amino groups of (hydroxy-)lysine residues leads to the formation of intra- and interhelical crosslinks [61]. The use of N-hydroxysuccinimide (NHS) has been reported to overall increase the crosslinking efficiency since it enables the formation of a more stable reactive ester during the reaction, as illustrated in Figure 3.33 [15,19,62]. In addition, to obtain a coupling rate of carboxyl groups higher than that exhibited by carboxylate anions, the pH of the reaction should be maintained over 5.8, that represents the pKa of collagen [61,63].

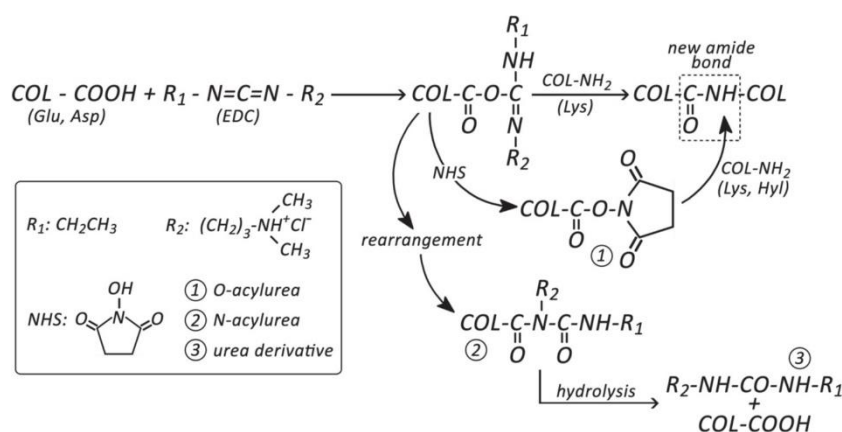


Figure 3. 33 Schematic representation of the chemical reactions occurring during the chemical crosslinking of collagen by means of EDC/NHS [62].

In this work, the chemical treatment was performed by incubating Coll/MBG_SG_Sr4% samples (as described in Section 2.2.5) in a solution of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-hydrochloride (EDC) and 25 mM N-hydroxysuccinimide (NHS) in 98% ethanol at 37 °C for 24 hours. In particular, the concentration and the ratio of EDC and NHS were selected, based on protocols present in the literature, to obtain the best crosslinking efficiency while preserving the cytocompatibility of the system, that can be hindered by higher concentrations of the crosslinker [32,64,65]. Moreover, some studies proved that the use of ethanol, alongside guarantying the proper dissolution of the chemical reagents, did not alter the helical structure of collagen and better prevented the hydrolysis of EDC [61].

After 24 hours of reaction, the samples were collected and washed with abundant distilled water to remove any crosslinker residuals and immediately imaged, as shown in Figure 3.34.

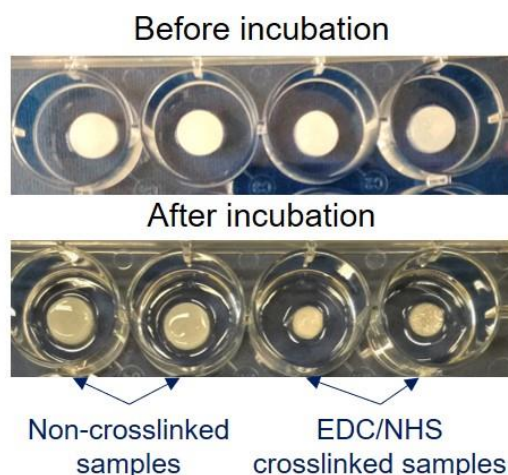


Figure 3. 34 Coll/MBG_SG_Sr4% sample shrinkage after EDC/NHS crosslinking.

The observation of Coll/MBG_SG_Sr4% before and after EDC/NHS chemical crosslinking highlighted the high shrinkage of the chemical treated samples, that reduced of almost half their diameter, as clearly visible in Figure 3.34. Besides the molecule crosslinking, the significant dimensional shrinking can be ascribed to the overall sample drying due to the use of ethanol as well as an incubation temperature of 37 °C, as previously observed [66].

Following the sample freeze drying, FE-SEM analysis was exploited to investigate any potential change of the morphological features at the micro- and nano-scale.

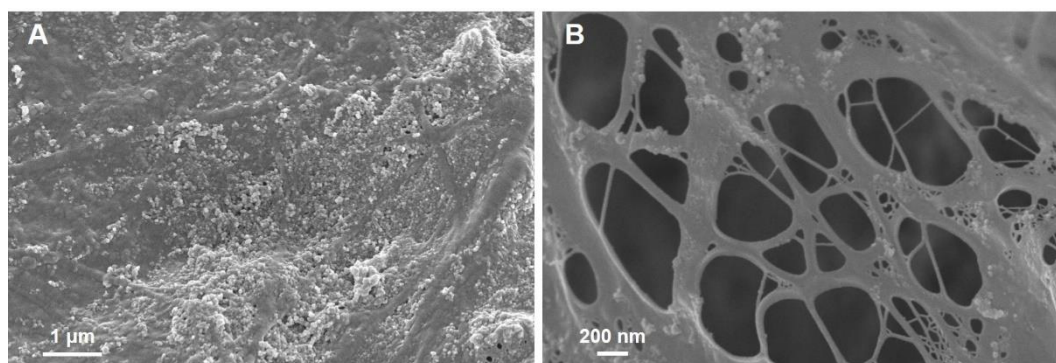


Figure 3. 35 Cross-sectional FE-SEM images showing Coll/MBG_SG_Sr4% samples after EDC/NHS crosslinking at different magnifications.

FE-SEM images of EDC/NHS crosslinked samples, represented in Figure 3.35, showed no significant changes in the micro and nano-organisation of the composite after the chemical treatment. The cross-sectional images demonstrated the presence of walls of collagen fibrils embedding MBG_SG_Sr4% particles (Figure 3.35B). However, in some areas of the sample, non-fibrillar ground substance was detected, probably related to low molecular collagen fragments which were not fully developed into macro-fibrils.

The creation of new chemical bonds and the overall chemical features of the crosslinked Coll/MBG_SG_Sr4% system were investigated by means of ATR-FTIR spectroscopy, where the resulting spectrum was compared to those of collagen and non-crosslinked system (Figure 3.36).

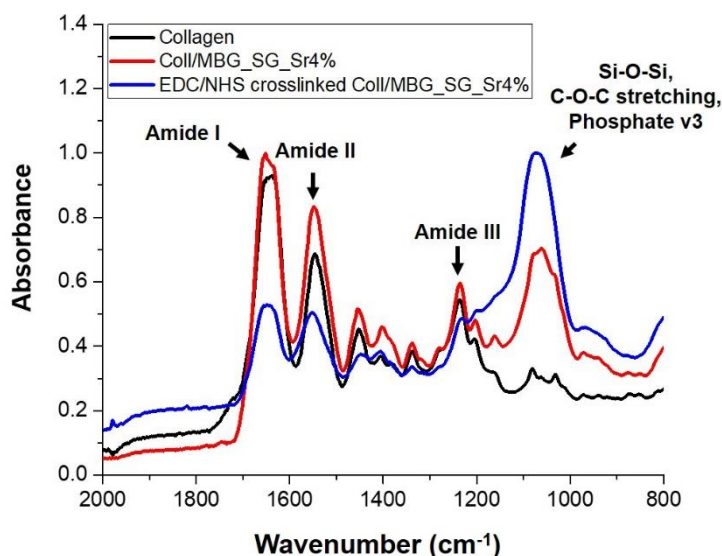


Figure 3. 36 ATR-FTIR spectra of Coll/MBG_SG_Sr4% before and after EDC/NHS crosslinking.

The resulting spectrum of the EDC/NHS crosslinked Coll/MBG_SG_Sr4% system showed a slight increase in the intensity of amide II peak (about 1550 cm⁻¹) with regard to amide I (about 1550 cm⁻¹), that can be ascribed to the consumption of amine groups to form amide bonds, confirming that the carbonyl groups on the collagen molecules are preferential reaction sites for the chemical crosslinking [32]. The chemical treated system also showed a larger band between 1200 cm⁻¹ and 1000 cm⁻¹ probably attributable to the presence of three peaks of the C–O–C stretching vibration at 1133 cm⁻¹, 1122 cm⁻¹ and 1069 cm⁻¹, according to previous studies [32,65,67], with maximum at 1072 cm⁻¹ also due to the Si-O-Si bonds of MBG_SG_Sr4% particles (Table 3.23).

Table 3. 23. ATR-FTIR characteristic peaks of Coll/MBG_SG_Sr4% before and after EDC/NHS crosslinking.

	Amide I (C=O stretching)	Amide II (NH stretching)	Carbonate ν3	Amide III (CN bending)	Si-O-Si, C-O-C stretching, phosphate ν3
Collagen	1640 cm⁻¹	1545 cm⁻¹	1451 cm⁻¹	1236 cm⁻¹	1081, 1032 cm⁻¹
Coll/MBG_SG_Sr4%	1645 cm⁻¹	1545 cm⁻¹	1451 cm⁻¹	1235 cm⁻¹	1061 cm⁻¹
EDC/NHS crosslinked Coll/MBG_SG_Sr4%	1646 cm⁻¹	1551 cm⁻¹	1447 cm⁻¹	1232 cm⁻¹	1072 cm⁻¹

The potential strengthening of the composite following the chemical treatment with EDC/NHS and thus the formation of stronger covalent bonds was investigated by means of dynamic amplitude sweep tests as reported in Figure 3.37A, while the denaturation temperature of the system was detected by means of a temperature ramp up to 80 °C (Figure 3.37B).

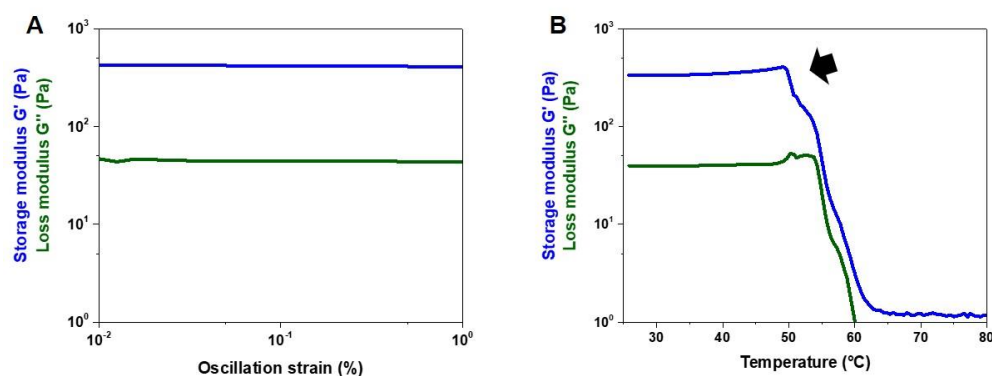


Figure 3. 37 Amplitude sweep (A) and temperature ramp (B) tests showing G' (storage modulus), G'' (loss modulus) values and denaturation temperature of Coll/MBG_SG_Sr4% after EDC/NHS crosslinking.

The storage modulus and loss modulus of the composite after the chemical treatment were registered to be about 418.1 ± 12.3 Pa and 44.6 ± 5.7 Pa, therefore similar to those reported for the non-crosslinked system (about 434.9 ± 64.3 Pa and 30.8 ± 15.6 Pa, respectively). Similarly, as shown in Figure 3.37B, the denaturation temperature of the material detected by the drop of G' and G'' was found to be about 48.6 °C, only slightly higher than the value obtained for the non-chemically treated system (45.1 °C).

Data resulting from rheological analyses thus suggested a poor crosslinking efficiency and almost no effects on the increase of the material stiffness and stability. According to the reported data, the slow increase in the material strength induced by EDC crosslinking was already observed in other studies [32].

The overall results reported thus proved that EDC/NHS crosslinking did not show any significant enhancement in the overall physico-chemical and mechanical properties of the collagenous system. Despite the large use of EDC as chemical crosslinker of collagen, its extremely low coupling efficiency has been already reported in different studies [15,58,61,66]. The low crosslinking density can be ascribed to the high tendency of EDC to hydrolysis, even in presence of NHS.

In contrast to other crosslinkers such as diol-related crosslinkers or glutaraldehyde characterised by a long-range effectiveness, EDC presented a limited efficiency mainly related to its nature of zero-length crosslinker. Based on these considerations, the coupling reaction can most likely occur inside the triple-helical structure, while inter-molecular and inter-fibrillar bonds are hindered [57]. Accordingly, most of the EDC/NHS treatments are usually performed on freeze-dried denser constructs, to favour the crosslinking reaction.

On the base of the results obtained, proving a significant shrinkage of the material and low mechanical stability following the chemical treatment, the use of

EDC/NHS as potential chemical crosslinking strategy for the stabilisation of the 3D printed scaffolds was discarded and no further explored.

3.5.2 Chemical crosslinking by 4-star poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG)

The use of 4-star poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG) was explored as alternative chemical crosslinking treatment to enhance the properties of the Coll/MBG_SG_Sr4% system. According to previous works in the literature, this chemical crosslinker was reported to increase the material stability with very low cytotoxic effects both *in vitro* and *in vivo* [24,26]. Moriarty N. and co-workers in fact showed that 4-StarPEG was successfully incorporated in the formulation of collagen-based hydrogel spheres for cell encapsulation without hindering cell metabolic activity and vitality [27].

Based on previous protocols, Coll/MBG_SG_Sr4% samples were incubated in 1 wt% 4-StarPEG in PBS solution at 37 °C for 24 h in order to allow the crosslinker diffusion and the further reaction with collagen functional groups. In detail, the crosslinking process involves the reaction between the succinimidyl groups of 4-StarPEG with the amine groups present on the collagen molecules and the further formation of intermolecular covalent bonds, as shown in Figure 3.38 [24].

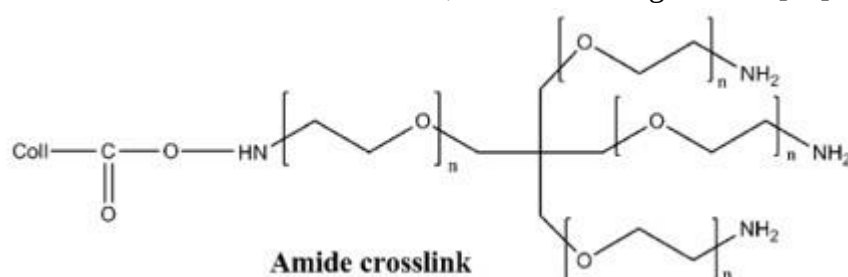


Figure 3. 38 Chemical crosslinking between collagen and 4-star poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG)

After the chemical treatment, samples were collected and washed in distilled water to remove any crosslinker residuals as well as the salt deposits due to the incubation in PBS, and further lyophilised to enable the morphological assessment.

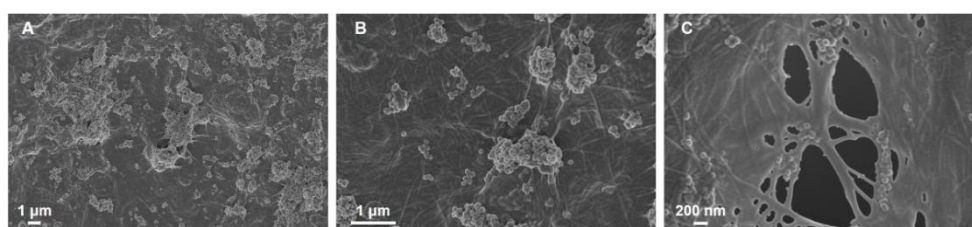


Figure 3. 39 FE-SEM images of Coll/nano-HA after 4-StarPEG crosslinking at different magnifications.

FE-SEM images, reported in Figure 3.39, clearly reported the homogenous distribution of the spherical nano-sized MBG particles into the fibrous collagen matrix following the sol–gel transition of the material at 37 °C and the further chemical crosslinking. At higher magnifications (Figure 3.39C), the distribution of the MBG_SG_Sr4% particles along the collagen fibres resembled biomimetic

structures. No evident changes in the morphological features of the chemically crosslinked composite were observed compared to the non-treated system samples.

The potential creation of covalent bonds induced by the chemical crosslinking with 4-StarPEG was assessed by means of ATR-FTIR analysis, reporting the spectrum of crosslinked Coll/MBG_SG_Sr4% together with those of collagen and the non-crosslinked system.

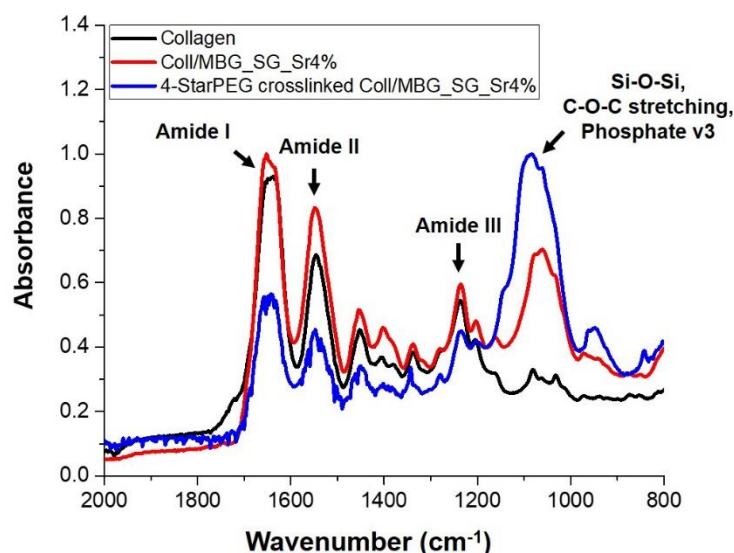


Figure 3.40 ATR-FTIR spectra of Collagen and Coll/MBG_SG_Sr4% before and after 4-StarPEG crosslinking.

The spectra reported in Figure 3.40 showed similar patterns for both non-crosslinked and crosslinked Coll/MBG_SG_Sr4% system, evidencing the typical bands of collagen and MBG particles, as summarised in Table 3.24.

No evident differences or shifts of the peaks were observed between the non-crosslinked and the crosslinked system. However, in the spectrum reported by the chemically treated system, the broadening of the peak with a maximum at 1061 cm^{-1} can be attributed to the formation and contribution of a new peak at about 1100 cm^{-1} due to the C–O–C stretching vibration of 4-StarPEG molecule [68].

Table 3.24 ATR-FTIR characteristic peaks of Collagen and Coll/MBG_SG_Sr4% before and after 4-StarPEG crosslinking.

	Amide I (C=O stretching)	Amide II (NH stretching)	Carbonate ν_3	Amide III (CN bending)	Si-O-Si, C-O-C stretching, Phosphate ν_3	Phosphate ν_1
Collagen	1640 cm^{-1}	1545 cm^{-1}	1451 cm^{-1}	1236 cm^{-1}	$1081, 1032\text{ cm}^{-1}$	-
Coll/MBG_SG_Sr4%	1645 cm^{-1}	1545 cm^{-1}	1451 cm^{-1}	1235 cm^{-1}	1061 cm^{-1}	970 cm^{-1}
4-StarPEG crosslinked Coll/MBG_SG_Sr4%	1646 cm^{-1}	1549 cm^{-1}	1453 cm^{-1}	1236 cm^{-1}	1082 cm^{-1}	$949, 842\text{ cm}^{-1}$

Alongside the physico-chemical properties, 4-StarPEG crosslinked Coll/MBG_SG_Sr4% samples were analysed by means of rheological tests to investigate the resulting mechanical and thermal strength. The biomaterial was subjected to amplitude sweep tests and temperature ramps as previously reported for the non-treated system.

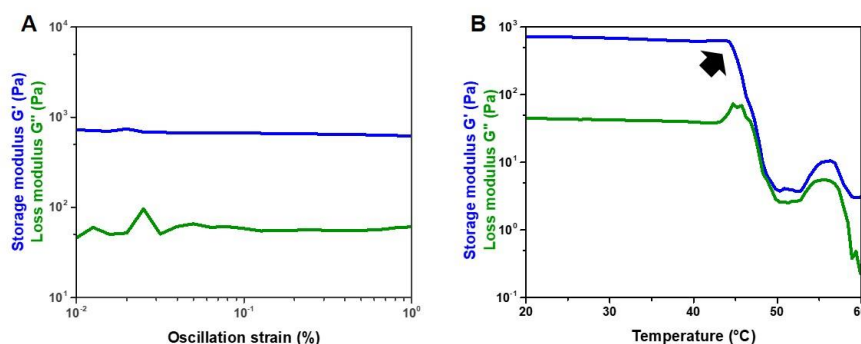


Figure 3. 41 Amplitude sweep (A) and temperature ramp (B) tests showing G' (storage modulus), G'' (loss modulus) values and denaturation temperature of Coll/MBG_SG_Sr4% after 4-StarPEG crosslinking.

Results reported in Figure 3.41 showed an increase in the storage and loss modulus with values of about 686.5 Pa and 55.1 Pa respectively, where G' increased of more than 50% compared to the non-crosslinked system. On the contrary, no differences were detected in the denaturation temperature of the material, with a resulting drop of G' and G'' at 44.7 °C, as evidenced in Table 3.25.

Table 3. 25 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of 4-StarPEG crosslinked Coll/MBG_SG_Sr4% compared to the data obtained from the non-crosslinked system.

	Storage modulus - averaged (G')	Loss modulus - averaged (G'')	Denaturation Temperature
4-StarPEG crosslinked Coll/MBG_SG_Sr4%	686.5 ± 61.3 Pa	55.1 ± 10.8 Pa	44.7 ± 0.5 °C
Coll/MBG_SG_Sr4% (non-crosslinked)	434.9 ± 64.3 Pa	30.8 ± 15.6 Pa	45.1 ± 0.9 °C

To further investigate the material stability, the enzymatic degradation of 4-StarPEG crosslinked and non-crosslinked samples in a concentrated collagenase solution was compared. In details, bacterial collagenase favours the specific hydrolytic cleavage of collagen molecules in non-polar regions. The cleavage mechanisms can act either in a single alpha-chain or simultaneously across three chains of the triple helix, leading to the protein denaturation [15].

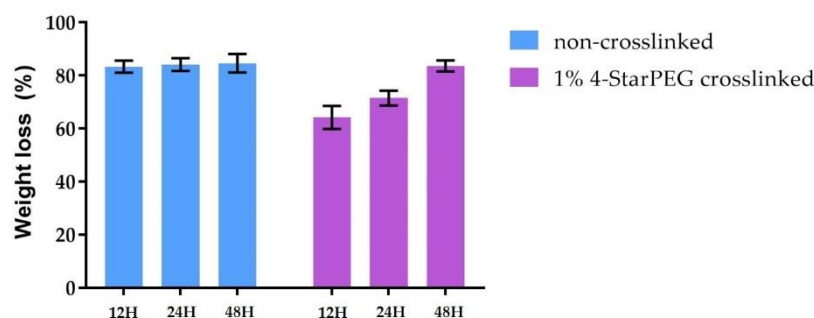


Figure 3. 42 Weight loss of 4-StarPEG crosslinked and non-crosslinked Coll/MBG_SG_Sr4% samples following incubation in collagenase up to 48 h.

As represented in Figure 3.42 and summarised in Table 3.26, the investigated samples lost most of their initial weight after 48 h when subjected to collagenase digestion. Even though 4-StarPEG crosslinked Coll/MBG_SG_Sr4% samples retained about 60% of the initial weight at 12 h of incubation, the detected degradation rate proved a poor effect of the chemical crosslinker on the material stability, as the measured values just slightly enhanced compared to the non-cross-linked reference.

Table 3. 26 Weight loss (%) of 4-StarPEG crosslinked and non-crosslinked Coll/MBG_SG_Sr4% samples following incubation in collagenase up to 48 h.

	Weight loss at 12H	Weight loss at 24H	Weight loss at 48H
Coll/MBG_SG_Sr4%	83.3% ± 2.3	84.1% ± 2.4	84.6% ± 3.5
4-StarPEG crosslinked Coll/MBG_SG_Sr4%	64.2% ± 4.3	71.5% ± 2.8	83.6% ± 2.1

The rheological assessments alongside the collagenase degradation tests were exploited to compare the properties of the hybrid system before and after the 4-StarPEG treatment and to detect any potential increase in its mechanical and thermal stability. However, the differences observed comparing the two systems were not very significant.

Considering the significant ion release from Sr-MBGs in saline solutions, observed in previous studies [10,36] and reported in Section 3.2.4 , the potential loss of strontium during and after the crosslinking treatment was monitored by means of ICP analysis, as shown in Figure 3.43. The average amount of strontium ion incorporated in each material sample was previously detected by ICP analyses after sample acidic digestion.

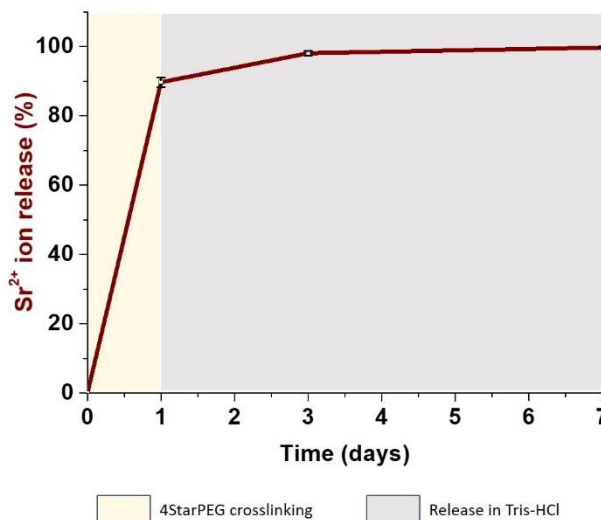


Figure 3. 43 Sr²⁺ ion release from 4-StarPEG crosslinked Coll/MBG_SG_Sr4% up to 7 days.

As reported for the non-chemically crosslinked system, the presence of the collagenous matrix enabled a more sustained release of the ions, dampening the burst release observed for MBG_SG_Sr4% alone, where of incubation almost the total amount of ions was released after 3 hours. However, a significant strontium release, higher than 80% of the entire amount was registered after the 24 hours incubation needed for the chemical treatment, while the remaining 10% was released in the following 48 hours of incubation in Tris-HCl buffer. The high amount of strontium loss during the crosslinking process was considered a negative aspect. In fact, a more sustained release of functional ions is highly desirable in order to extend the pro-osteogenic effect of the biomaterial.

Table 3. 27 Sr²⁺ release values (%) from 4-StarPEG crosslinked Coll/MBG_SG_Sr4% at different time points (24h, 3d, 7d).

	24h	3d	7d
Sr ²⁺ release from 4-StarPEG crosslinked Coll/MBG_SG_Sr4%	89.7% ± 1.4	98.1% ± 0.6	99.7% ± 1.3

The reported results thus suggested an overall low efficiency of the selected crosslinking method, probably linked to low diffusion kinetics in the water-containing polymeric matrix, whereas higher crosslinking efficiency was obtained when using 4-StarPEG on lyophilized matrices. In such cases, the authors reported high resistance to enzymatic degradation in addition to a significant reduction of free amino content after 4-StarPEG crosslinking, proving the effective enhancement of the material stability [24,26]. However, the mechanical strength of 4-StarPEG crosslinked collagen resulted to be weaker when compared to matrices crosslinked with alternative methods such as genipin and glutaraldehyde [24,38]. Considering the potential high cytocompatibility reported by some studies [69,70],

the biological assessment of 4-StarPEG crosslinked Coll/MBG_SG_Sr4% was further assessed, as described in Chapter 5.

However, based on the low efficiency of the crosslinking as well as the significant loss of strontium ions detected during the chemical treatment, the 4-StarPEG was not exploited to enhance the stability of the 3D printed scaffolds.

3.5.3 Genipin crosslinking

The use of genipin, a naturally derived chemical crosslinker extracted from gardenia fruit (*Gardenia jasminoides* Ellis), has been widely reported to improve both the stiffness and the thermal response of collagen matrices, also showing lower toxicity levels compared to alternative and more common synthetic crosslinking agents [32,60,69,71,72].

According to Kim et al. [73], genipin causes crosslinking of free amino groups of collagen through the formation of cyclic structures, which act as intra- and inter-molecular bridges along the fibers.

In the present work, the genipin-based crosslinking approach was thus exploited to improve the overall stiffness and stability of the collagen-based systems in view of their effective use as bone scaffolds.

A large number of studies on the use of genipin as a collagen crosslinker usually reports the dissolution of the natural agent in aqueous solutions and the subsequent incubation of the material, where longer times and a physiological temperature of 37 °C demonstrated the best results in terms of crosslinking efficiency [60,72,74–76]. Despite most of the studies reported the combination of genipin with aqueous-based systems, the previous results obtained with 4-StarPEG crosslinked Coll/MBG_SG_Sr4% showed that the use of saline solutions can lead to a premature abundant loss of the strontium ions present in the material formulation.

Starting from these data, and considering the high solubility of genipin in ethanol, the use of genipin as chemical crosslinker of collagen was explored both in combination with PBS and ethanol solutions. The crosslinking of Coll/MBG_SG_Sr4% in the two different media (GEN/PBS and GEN/EtOH) was assessed by observing the extent of strontium ion loss during the treatment, as well as the resulting mechanical and biological features. As described in Section 2.2.5, 0.5 wt% genipin in PBS solution (GEN/PBS) and 0.5 wt% genipin in 70% ethanol solution (GEN/EtOH) were exploited to promote the formation of inter- and intra-molecular covalent bonds, as schematically illustrated in Figure 3.44.

After the incubation in the crosslinking solutions at 37 °C for 24 hours, the material samples presented an intense blue colour due to the increased fluorescence intensity induced by genipin crosslinking without showing evident shrinkage, as observed in other studies [73]. The samples were subsequently washed in abundant distilled water in order to remove any residuals of unreacted chemical crosslinkers, and further analysed.

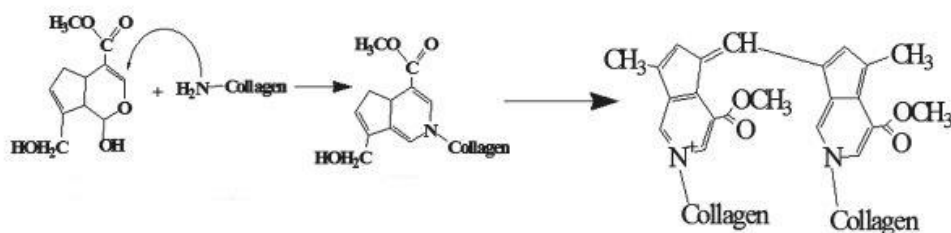


Figure 3. 44 Schematic illustration of the chemical crosslinking of collagen with genipin [76].

Firstly, FE-SEM was used to investigate the micro- and nano-architectures of the Coll/MBG_SG_Sr4% system after the chemical treatments with GEN/EtOH and GEN/PBS solutions, as shown in Figure 3.45.

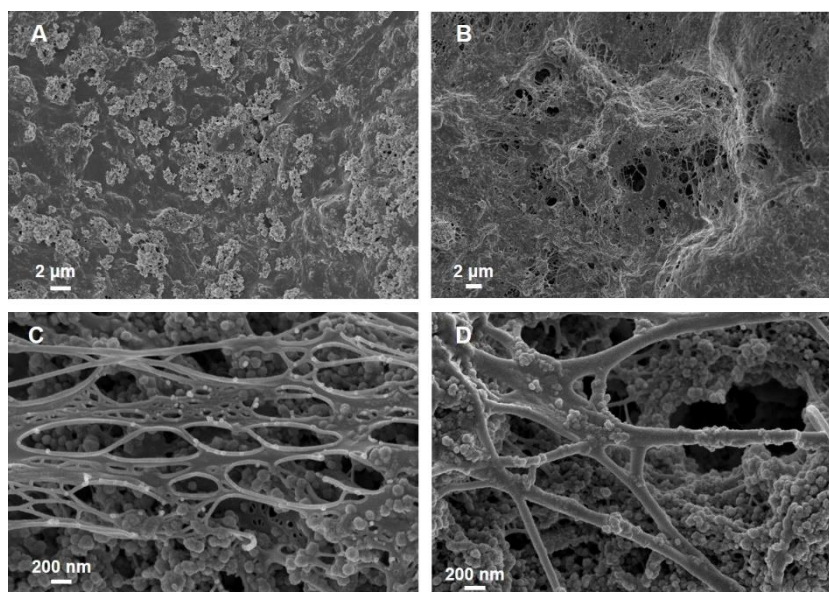


Figure 3. 45 FE-SEM images of GEN/PBS (A, C) and GEN/EtOH (B, D) crosslinked Coll/MBG_SG_Sr4% at different magnifications.

FE-SEM images showed well-packed nano-fibril collagenous matrices embedding MBG_SG_Sr4%, with no evident particle agglomerates. The morphological assessment did not prove any significant change of the material organisation after the chemical crosslinking treatments. However, compared to GEN/PBS crosslinking, the treatment with GEN/EtOH seemed to promote a higher amount of fibrillar areas instead of an amorphous denser matrix. In some regions, better dispersion of MBG_SG_Sr4% promoted their arrangement along collagen fibrils, nicely resembling the arrangement of the mineral phase within the native bone matrix (Figure 3.46). As previously reported, this is considered an important factor, since the creation of biomimetic nanostructured systems has proved to beneficially influence not only stem cells osteogenic differentiation but also to accelerate the mineralization kinetics *in vivo* [33,34,77,78].

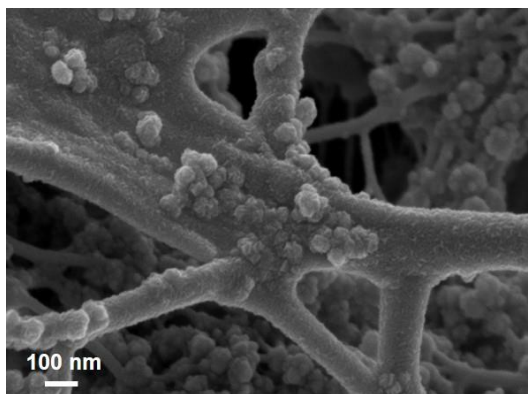


Figure 3. 46 FESEM image of GEN/EtOH crosslinked Coll/MBG_SG_Sr4% showing the embedding of inorganic particles into collagen

The potential chemical reactions during the crosslinking treatment were investigated by means of ATR-FTIR. Due to the similarity of the results, only the spectrum of GEN/EtOH crosslinked Coll/MBG_SG_Sr4% was reported in the graph shown in Figure 3.47.

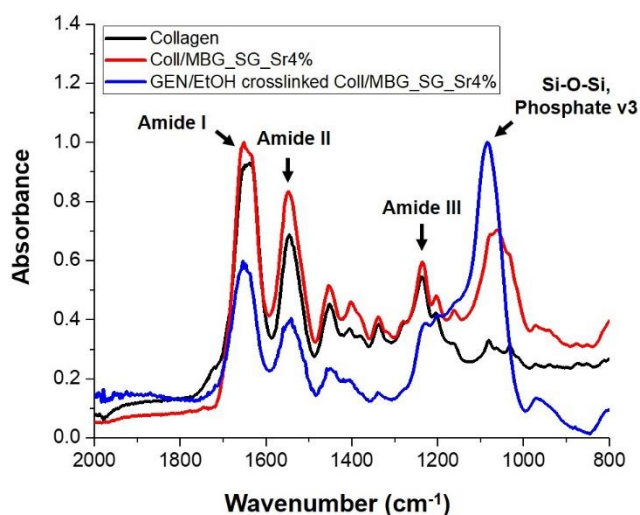


Figure 3. 47 ATR-FTIR spectra of Coll/MBG_SG_Sr4% before and after GEN/EtOH crosslinking.

According to the studies of Kim and co-workers [73], the spectrum of the genipin molecule is characterised by three main peaks centred at 1635 cm^{-1} , 1080 cm^{-1} and 990 cm^{-1} , corresponding to the C=C stretching, C-H in-plane bending and C-H out-of-plane bending, respectively. Based on these observations, the spectrum of the genipin-crosslinked Coll/MBG_SG_Sr4% system showed an evident broad peak in the range of $1200 - 1100\text{ cm}^{-1}$ and centred at about 1084 cm^{-1} , that can be indicative of the effective chemical reaction between genipin and collagen alongside the Si-O-Si contribution of MBG particles.

In addition, a slight shift of the amide I peak towards higher wavenumbers can be ascribed to the contribution at 1740 cm^{-1} of the ester group of C-O-O from genipin (Figure 3.44).

As observed in other studies, the genipin crosslinking starts from the formation of nitrogeniridoid derived by the nucleophilic substitution of the amine group in the

collagen structure on the C3 carbon of genipin. The subsequent replacement of the genipin's oxygen with the collagen's tertiary nitrogen leads to the formation of connecting cyclical structures. The two iridoids successively dimerize via a radical reaction creating a strong stable bond between collagen molecules [73].

Table 3. 28 ATR-FTIR characteristic peaks of Coll/MBG_SG_Sr4% before and after GEN/EtOH crosslinking.

	Amide I (C=O stretchin g)	Amide II (NH stretchin g)	Carbonat e v3	Amide III (CN bending)	Si-O-Si, Phosphat e v3
Collagen	1640 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1236 cm ⁻¹	1081, 1032 cm ⁻¹
Coll/MBG_SG_Sr4 %	1645 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1235 cm ⁻¹	1033 cm ⁻¹
GEN/EtOH crosslinked Coll/MBG_SG_Sr4 %	1654 cm ⁻¹	1545 cm ⁻¹	1451 cm ⁻¹	1230 cm ⁻¹	1084 cm ⁻¹

The long-time incubation in the crosslinking medium required for the effective stabilisation of the material can largely affect the release of osteogenic strontium ions from the system. For this reason, the release of Sr²⁺ ions from the Coll/MBG_SG_Sr4% system and the potential variation induced by the chemical crosslinking agent was analysed by ICP and reported in Figure 3.48. To assess the strontium released during the crosslinking treatment, the ion concentration in the supernatants collected after 10 and 24 h of contact with genipin solutions was measured. After 24 h, the crosslinked samples were transferred in Tris-HCl buffer (releasing medium) to verify the release of the remaining strontium ions. The percentage of ion released was calculated by considering the amount of strontium initially incorporated into MBG_SG_Sr4% particles (as measured by ICP on acid-digested samples) and the content of the inorganic phase in the hybrid formulation, assuming the homogeneous distribution of the particles.

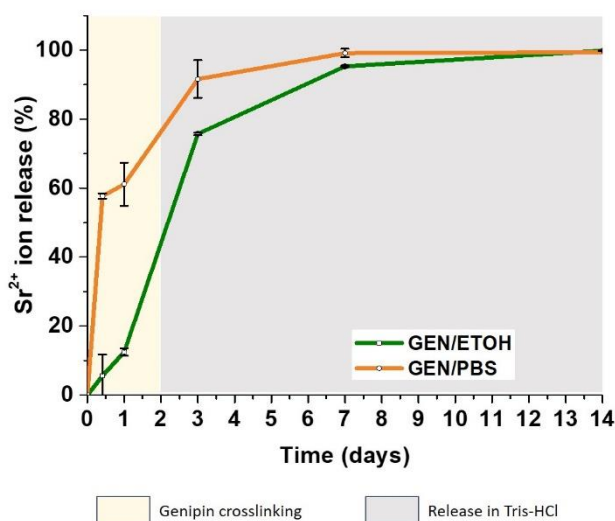


Figure 3. 48 Sr^{2+} ion release from GEN/EtOH and GEN/PBS crosslinked Coll/MBG_SG_Sr4% system.

The crosslinking process caused a significant release of Sr^{2+} ions when Coll/MBG_SG_Sr4% samples were treated with GEN/PBS solution, as reported in Table 3.29. In detail, GEN/PBS crosslinked samples displayed fast release kinetics reporting 57.7% and 61.2% of release, after 10 and 24 h, respectively (yellow zone, Figure 3.48). On the contrary, the use of GEN/EtOH solution presented a significant lower ion release during the crosslinking reaction time, showing a release of only 5.6% and 12.5% (yellow zone, Figure 3.48). Once soaked in the Tris-HCl buffer, both GEN/PBS and GEN/EtOH crosslinked material samples released almost the whole strontium incorporated ions after seven days, registering values of 99.1% and 95.3%, respectively.

Therefore, release profiles for Coll/MBG_SG_Sr4% samples crosslinked with GEN/PBS showed a rather high percentage of strontium ions released in the collected crosslinking solution after 24 h and the remaining amount totally was released after incubation in Tris-HCl within seven days. At variance, the use of GEN/EtOH showed a significant reduction of released ions during the crosslinking reaction time, registering significantly lower percentages, overall showing a slower release kinetics up to seven days, probably thanks to the reduced water content of the crosslinked system. The two crosslinking treatments and the chosen media proved to significantly influence the ion loss kinetics.

Table 3. 29 Sr^{2+} release values (%) from GEN/EtOH and GEN/PBS crosslinked Coll/MBG_SG_Sr4% system.

	10h	24h	3d	7d
GEN/PBS crosslinked Coll/MBG_SG_Sr4%	57.7% ± 0.8	61.2% ± 6.2	91.6% ± 5.4	99.1% ± 1.2
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	5.6% ± 3.1	12.5% ± 1.1	75.7% ± 1.3	95.3% ± 2.2

Finally, the effect of the chemical crosslinking with GEN/PBS and GEN/EtOH on Coll/MBG_SG_Sr4% system was investigated through rheological tests, particularly observing the increase of material strength and its thermal stability, as shown in Figure 3.49.

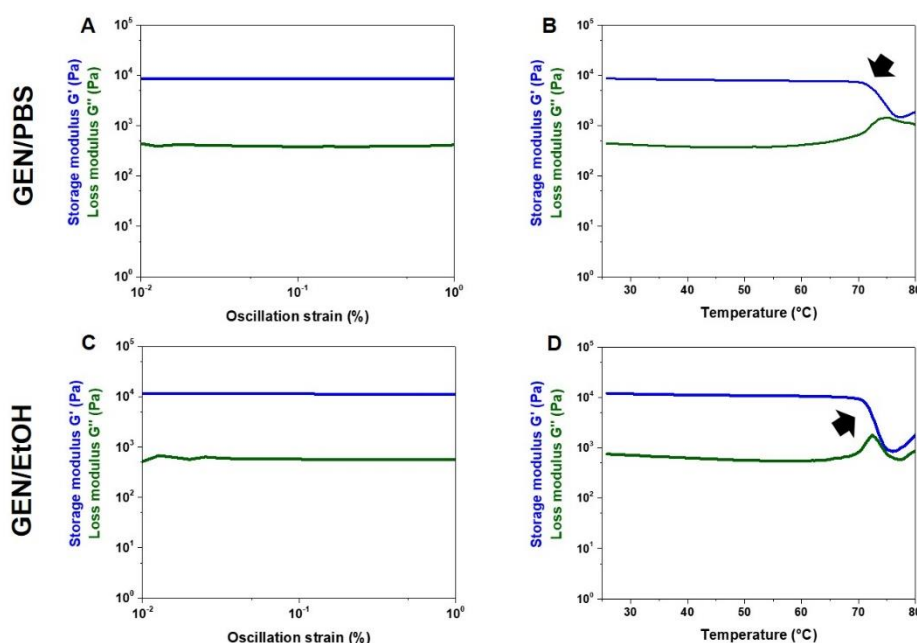


Figure 3. 49 Amplitude sweep tests (A, C) and temperature ramp (B, D) performed on Coll/MBG_SG_Sr4% samples crosslinked with GEN/PBS and GEN/EtOH.

As represented in Figure 3.49A, amplitude sweep tests performed at 37 °C after treating Coll/MBG_SG_Sr4% system in GEN/PBS solution gave mean values of G' and G'' of about 8690 Pa and 391 Pa, respectively. The hybrid formulation crosslinked by GEN/EtOH solution showed even higher strength, registering mean values of G' and G'' of about 11032 Pa and 599 Pa in the same range of strains (Figure 3.49 C). The relevant gap between G' and G'' obtained after genipin crosslinking is indicative of a predominant elastic behaviour, different from the non-crosslinked system.

Irrespective of the use of GEN/PBS or GEN/EtOH, the chemical crosslinking of collagen induced a significant increase in the material denaturation temperature, as clearly reported in Figure 3.49 and Table 3.30. Genipin-crosslinked Coll/MBG_SG_Sr4% subjected to temperature ramp in the 25–80 °C range underwent a sharp decrease of the overall complex modulus of the material at about 70 °C, indicative of the structural collapse.

Table 3. 30 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of GEN/PBS and GEN/EtOH crosslinked Coll/MBG_SG_Sr4% compared to the non-crosslinked system.

	Storage modulus - averaged (G')	Loss modulus - averaged (G'')	Denaturation Temperature
GEN/PBS crosslinked Coll/MBG_SG_Sr4%	8690.3 ± 88.9 Pa	391.6 ± 33.5 Pa	70.4 ± 0.2 °C
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	11032.7 ± 99.4 Pa	599.4 ± 65.1 Pa	70 ± 0.5 °C
Coll/MBG_SG_Sr4% (non- crosslinked)	434.9 ± 64.3 Pa	30.8 ± 15.6 Pa	45.1 ± 0.9 °C

The successful chemical crosslinking of Coll/MBG_SG_Sr4% was thus confirmed by the significant increase of the related viscoelastic properties, as well as the enhanced thermal stability both in the case of GEN/PBS and GEN/EtOH treatment. The important increase of the storage modulus values, in addition to the wide gap between the storage modulus and the loss modulus, proved the evident prevailing of the elastic response, leading to the formation of fully developed solid structures. Furthermore, the chemical crosslinking strongly influenced the stability of the complex fibrillar structure of collagen, as resulted from the temperature ramp tests. In detail, the denaturation temperature of collagen related to the helix-to-coil-transition of fibrils strongly increased up to 70 °C after the genipin treatment, leading to enhanced thermo-responsive properties of the hybrid system. Even if relevant differences were not registered in terms of denaturation temperature, the treatment with GEN/EtOH led to greater values of the overall complex modulus of the material, achieving higher stiffness compared to the GEN/PBS crosslinked samples, potentially due to the partial material dehydration and the consequent reduced water content.

Hydrolytic and enzymatic degradation of Coll/MBG_SG_Sr4% before and after GEN/PBS and GEN/EtOH crosslinking was investigated to further prove the stability of the system both in aqueous and enzymatic solutions, as represented in Figure 3.50.

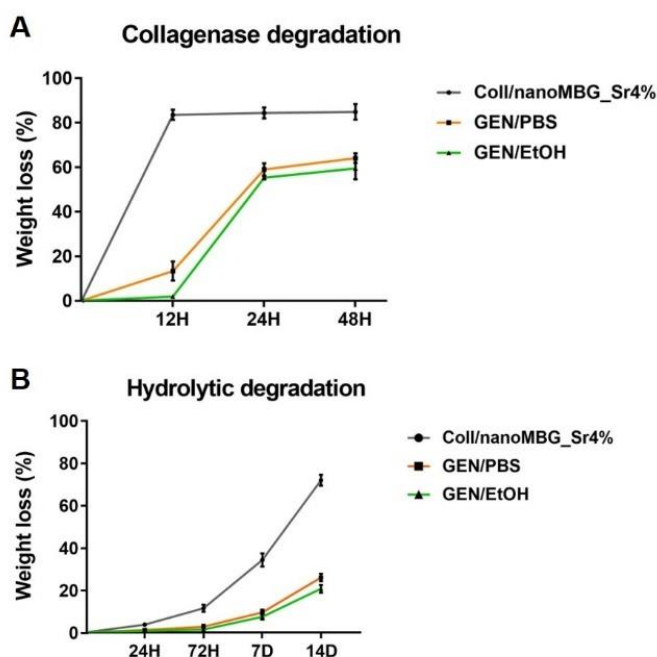


Figure 3.50 Enzymatic (A) and hydrolytic (B) degradation of the Coll/MBG_SG_Sr4% system before and after GEN/PBS and GEN/EtOH treatment.

The collagenase digestion of both crosslinked and non-crosslinked samples at 37 °C was investigated by measuring the percentage of weight loss up to 48 h of soaking in the enzymatic solution. As illustrated in Figure 3.50A, the degradation process of non-crosslinked Coll/MBG_SG_Sr4% samples showed a rapid weight loss, retaining less than 20% of the initial weight already after 12 h of incubation into the collagenase solution. On the contrary, both GEN/PBS and GEN/EtOH crosslinked samples retained about 40% of the initial weight after 48 h of incubation, confirming the efficacy of the chemical treatment.

Table 3.31 Weight loss (%) of GEN/PBS and GEN/EtOH crosslinked and non-crosslinked Coll/MBG_SG_Sr4% samples following incubation in collagenase up to 48 h.

	Weight loss at 12H	Weight loss at 24H	Weight loss at 48H
Coll/MBG_SG_Sr4%	83.3 ± 2.3	84.1 ± 2.4	84.6 ± 3.5
GEN/PBS crosslinked Coll/MBG_SG_Sr4%	13.3 ± 4.3	58.8 ± 2.8	63.9 ± 2.1
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	1.9 ± 0.1	55.1 ± 0.7	59.3 ± 4.9

In parallel, the hydrolytic degradation of Coll/MBG_SG_Sr4% before and after genipin treatment was explored up to 14 days of incubation in PBS at 37 °C.

Similar to the previous results, the non-crosslinked system showed less stability, retaining less than 30% of the initial weight after 14 days, as shown in Figure 3.50B. On the contrary, both GEN/PBS and GEN/EtOH showed high

stability in the aqueous environment, registering less than 30% of weight loss at 14 days.

Table 3. 32 Weight loss (%) of GEN/PBS and GEN/EtOH crosslinked and non-crosslinked Coll/MBG_SG_Sr4% samples following hydrolytic degradation up to 14 days.

	Weight loss at 24H	Weight loss at 72H	Weight loss at 7D	Weight loss at 14D
Coll/MBG_SG_Sr4%	3.7 ± 1.7	11.6 ± 1.7	34.7 ± 3.2	72.9 ± 2.6
GEN/PBS crosslinked Coll/MBG_SG_Sr4%	1.1 ± 0.2	2.7 ± 0.9	9.6 ± 1.3	26.3 ± 1.8
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%	0.7 ± 0.1	1.4 ± 0.7	7.5 ± 1.4	20.9 ± 1.9

As previously reported, during the enzymatic degradation, collagenase cleaves two of three helical chains of collagen in specific non-polar regions, resulting in the final fibril disintegration (Figure 3.51) [9,15]. The rapid weight loss of non-crosslinked samples reported in Figure 3.50 resulted from the weak intermolecular interactions between collagen chains. On the contrary, the formation of covalent bonds upon genipin treatment was confirmed by the observed gradual weight loss for both GEN/PBS and GEN/EtOH crosslinked Coll/MBG_SG_Sr4% samples, due to a slower degradation of crosslinked structures, and further confirmed by the longer stability of the system in aqueous solutions.

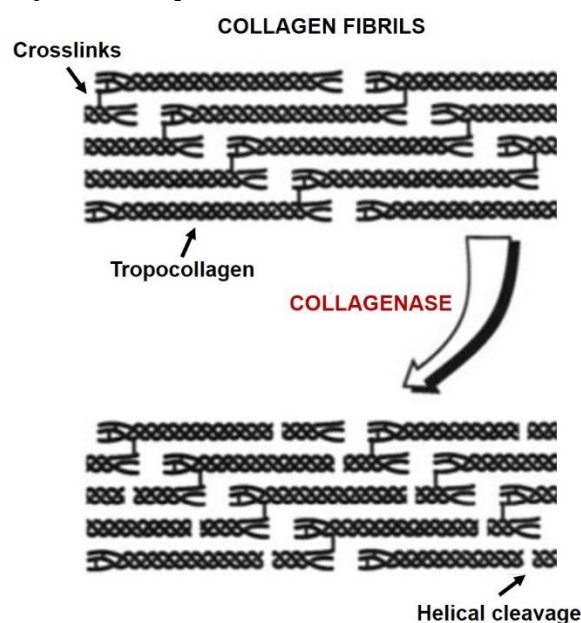


Figure 3. 51 Mechanism of collagen digestion by collagenase

Based on the promising results obtained by the genipin chemical treatment on the Coll/MBG_SG_Sr4%, this crosslinking strategy was explored on Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA. Despite the similar results obtained between GEN/PBS and GEN/EtOH, the latter was selected particularly for the opportunity

to preserve almost the total amount of strontium during the chemical treatment, as well as for the better results obtained in terms of material stiffness and stability.

The chemical crosslinking of Coll/nano-HA was thus investigated by using a solution of 0.5 wt% genipin in 70% ethanol, observing the influence over the final physico-chemical and mechanical properties of the system.

As previously reported and described in Section 2.2.5, material samples were incubated in the crosslinking medium at 37 °C for 24 hours and subsequently washed to remove the crosslinker residuals.

The morphological assessment by FE-SEM was performed on cross-sections of lyophilised samples and the resulting images are reported in Figure 3.52.

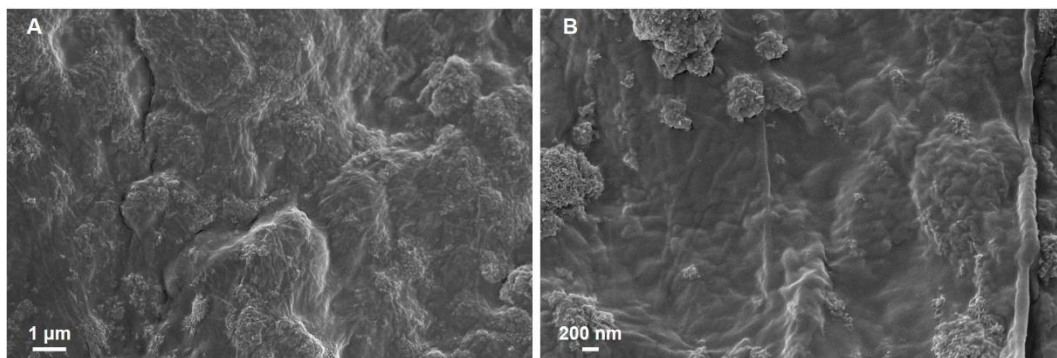


Figure 3. 52 Cross-sectional FE-SEM images showing GEN/EtOH crosslinked Coll/nano-HA samples at different magnifications.

FE-SEM images proved the homogeneous distribution of nano-HA particles embedded in the fibrillar collagenous matrix, as observed for the non-crosslinked system samples. In general, no significant changes in the micro and nanostructure were detected after the chemical treatment, as previously observed by the MBG containing system.

The potential chemical reactions between collagen molecules induced by the genipin treatment was investigated by means of ATR-FTIR as shown in Figure 3.53, where the spectrum of Coll/nano-HA was registered before and after the genipin crosslinking.

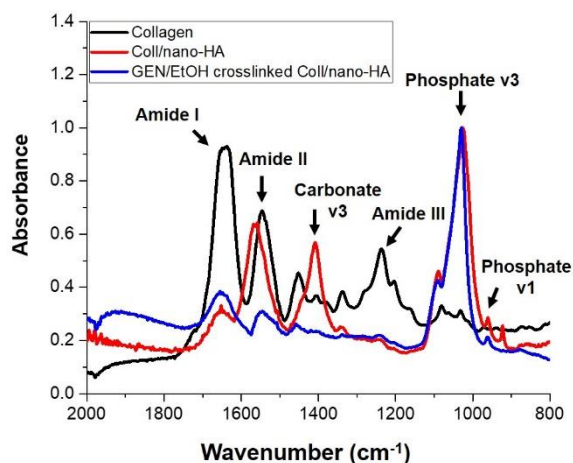


Figure 3. 53 ATR-FTIR spectra of Collagen and Coll/nano-HA before and after GEN/EtOH crosslinking.

The resulting spectrum of genipin crosslinked Coll/nano-HA resulted in the significant reduction of the contributes of amide I, II and III of collagen (1652 cm^{-1} , 1546 cm^{-1} , 1242 cm^{-1} respectively), where the band of amide III almost disappeared. In addition, a slight shift in the phosphate vibration bands centred at 1090 cm^{-1} and 1031 cm^{-1} towards higher wavenumbers were observed after the chemical treatment, as reported in Table 3.33.

Table 3. 33 ATR-FTIR characteristic peaks of Collagen and Coll/nano-HA before and after GEN/EtOH crosslinking.

	Amide I (C=O stretchi ng)	Amide II (NH stretchi ng)	Carbon ate v3	Amide III (CN bendin g)	Phosph ate v3	Phosph ate v3	Phosph ate v1
Collagen	1640 cm^{-1}	1545 cm^{-1}	1404 cm^{-1}	1236 cm^{-1}	1081 cm^{-1}	1031 cm^{-1}	-
Coll/nano-HA	1649 cm^{-1}	1563 cm^{-1}	1407 cm^{-1}	1240 cm^{-1}	1088 cm^{-1}	1025 cm^{-1}	960 cm^{-1}
GEN/EtOH crosslinked Coll/nano-HA	1652 cm^{-1}	1546 cm^{-1}	1457 cm^{-1}	1242 cm^{-1}	1090 cm^{-1}	1031 cm^{-1}	961 cm^{-1}

To further explore the influence of genipin crosslinking over the final properties of the Coll/nano-HA system, rheological analyses were conducted on crosslinked samples and compared to the values obtained for the non-crosslinked material.

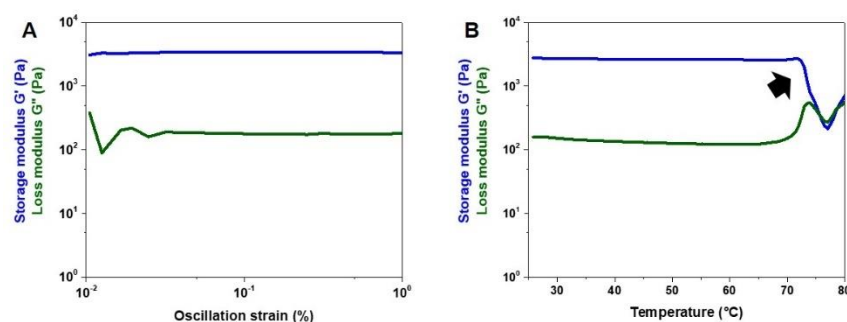


Figure 3. 54 Amplitude sweep tests (A) and temperature ramp (B) performed on GEN/EtOH crosslinked Coll/nano-HA samples.

Figure 3.54A showed the storage modulus (G') and loss modulus (G'') resulting from the dynamic amplitude sweep test on crosslinked samples performed between 0.01 and 1% strain at a constant frequency of 1 Hz. The obtained values, reported in Table 3.34, showed a significant increase of the material stiffness with G' and G'' measuring about 3215.7 Pa and 229.8 Pa respectively. In parallel, the temperature ramp performed up to 80 °C showed a drop in the visco-elastic properties of the material at about 73.4 °C.

These data thus suggested the effective chemical crosslinking of the collagen-based matrix, where G' increased of more than 3 times compared to the non-crosslinked system ($G' = 1316$ Pa) and the denaturation temperature rose from about 45 °C to 73 °C.

However, when compared to the MBG containing system, the resulting enhancement of the stiffness and thermal stability of the material was lower. As previously reported, collagen and hydroxyapatite prevalently form strong interactions between calcium ions and carboxylate groups of collagen, according to different studies in the literature [39,50–53,73] As a consequence, lower interactions between genipin and collagen molecules are favoured compared to the Coll/MBG_Sr4% system, also resulting in a lower degree of crosslinking.

Table 3. 34 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of GEN/EtOH crosslinked Coll/nano-HA.

	Storage modulus (G')	Loss modulus (G'')	Denaturation Temperature
GEN/EtOH crosslinked Coll/nano-HA	3215.7 ± 73.9 Pa	229.8 ± 27.8 Pa	73.4 ± 0.2 °C
Coll/nano-HA (non-crosslinked)	1316.1 ± 56.9 Pa	205.2 ± 21.3 Pa	45 ± 0.4 °C

The stability of the genipin crosslinked Coll/nano-HA was further assessed by means of enzymatic and hydrolytic degradation tests, as reported in Figure 3.55.

The enzymatic degradation was induced by incubating the material samples before and after chemical crosslinking in a collagenase solution at 37 °C up to 48

hours. On the contrary, the hydrolytic degradation was investigated by incubating the samples in PBS at 37 °C up to 14 days.

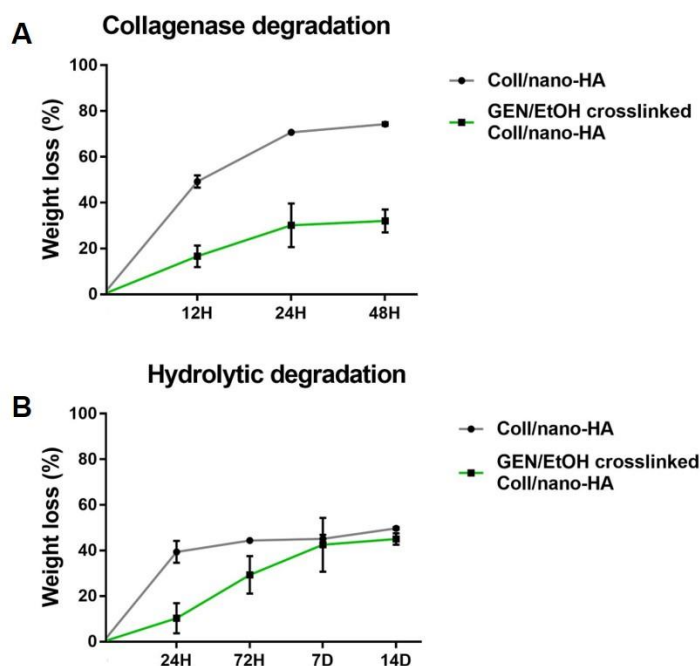


Figure 3.55 Enzymatic (A) and hydrolytic (B) degradation of GEN/EtOH crosslinked Coll/nano-HA.

At the different time points the percentage of weight loss was measured comparing with the original weight of samples, as summarised in Table 3.35 and 3.36.

The collagenase digestion of Coll/nano-HA samples showed a rapid weight loss of non-crosslinked samples, retaining about 50% of the initial weight after 12 h of incubation. On the contrary, GEN/EtOH crosslinked samples retained almost 70% of the initial weight after 48 h, confirming the efficacy of the chemical treatment.

Table 3.35 Weight loss (%) of GEN/EtOH crosslinked and non-crosslinked Coll/nano-HA samples following incubation in collagenase up to 48 h.

	Weight loss at 12H	Weight loss at 24H	Weight loss at 48H
Coll/nano-HA	49.2 ± 2.7	70.7 ± 1.3	74.3 ± 0.8
GEN/EtOH crosslinked Coll/nano-HA	16.6 ± 4.7	30.1 ± 9.5	32.0 ± 5.3

Accordingly, the hydrolytic degradation of Coll/nano-HA showed lower kinetics compared to the enzymatic digestion of the material samples.

As previously observed, the non-crosslinked system showed faster degradation kinetics over time, retaining about 50% of the initial weight after 14 days. Despite the GEN/EtOH crosslinked system showed higher stability in the aqueous environment up to 3 days, similar values of weight loss were reported for both non-

crosslinked and genipin-crosslinked Coll/nano-HA at 7 and 14 days of incubation. However, in both cases, more than 50% of the initial weight was retained, proving a good resistance of the material to degradation.

Table 3. 36 Weight loss (%) of GEN/EtOH crosslinked and non-crosslinked Coll/nano-HA samples following hydrolytic degradation up to 14 days.

	Weight loss at 24H	Weight loss at 72H	Weight loss at 7D	Weight loss at 14D
Coll/nano-HA	39.4 ± 4.8	44.4 ± 0.6	45.1 ± 1.8	49.7 ± 0.6
GEN/EtOH crosslinked Coll/nano-HA	10.3 ± 6.6	29.3 ± 8.2	42.5 ± 11.8	45.0 ± 2.5

The enhancement of the visco-elastic properties as well as the stability of the Coll/nano-HA system after chemical crosslinking with genipin, further proved the efficacy of the chemical treatment and its suitability for the stabilisation of scaffolds based on the collagen-based materials.

Finally, the genipin crosslinking was tested on the Coll/MBG_SG_Sr4%/nano-HA system. According to the optimised procedure, Coll/MBG_SG_Sr4%/nano-HA samples were immersed in GEN/EtOH solution at 37 °C for 24 hours.

The morphological features of the system at the micro and nano-structure after the chemical treatment were investigated by means of FE-SEM, as reported in Figure 3.56.

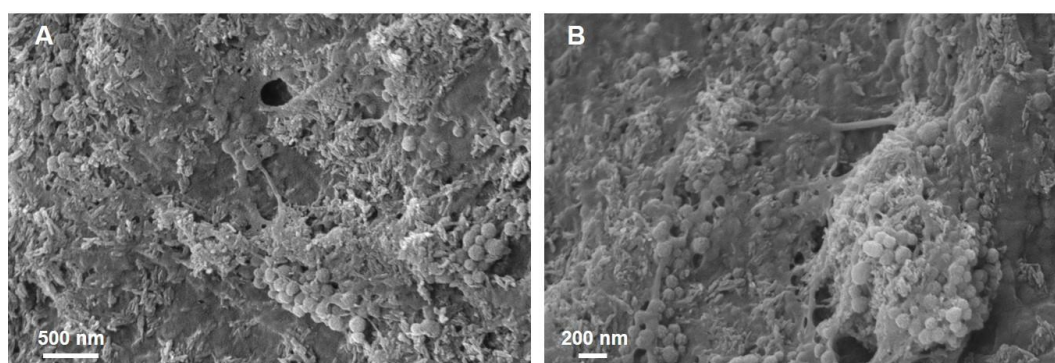


Figure 3. 56 Cross-sectional FE-SEM images showing GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA samples at different magnifications.

Similarly to the non-crosslinked samples, a dense fibrillar structure was observed after the genipin treatment. The homogeneous distribution of both MBG_SG_Sr4% and nano-HA particles was clearly visible and significant agglomerates were not detected even at lower magnifications.

The chemical features of Coll/MBG_SG_Sr4%/nano-HA were explored by means of ATR-FTIR analysis, where Figure 3.57 compared the spectra obtained from collagen and the system before and after genipin crosslinking.

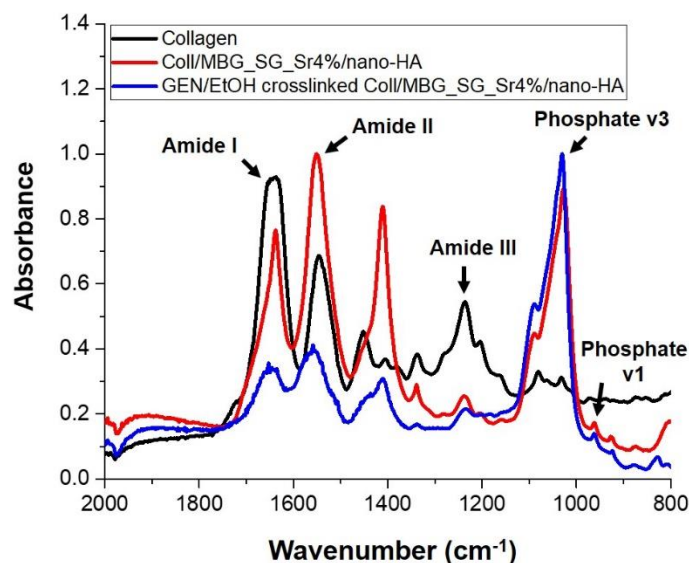


Figure 3. 57 ATR-FTIR spectra of Collagen and Coll/MBG_SG_Sr4%/nano-HA before and after GEN/EtOH crosslinking

The effective chemical crosslinking by genipin was suggested mainly observing the lower contribution of the bands related to amide I, II and III of collagen (1652 cm^{-1} , 1557 cm^{-1} , 1235 cm^{-1}), where the latter almost disappeared in the spectrum of crosslinked samples. In addition, as previously observed for the Coll/MBG_SG_Sr4% system, the shift of the amide I peak towards higher wavenumbers can be ascribed to the contribution at 1740 cm^{-1} of the ester group of C–O–O from genipin.

Table 3. 37 ATR-FTIR characteristic peaks of Collagen and Coll/MBG_SG_Sr4%/nano-HA before and after GEN/EtOH crosslinking

	Amide I (C=O stretchi ng)	Amide II (NH stretchi ng)	Carbon ate v3	Amide III (CN bendin g)	Phosph ate v3	Si-O- Si, Phosph ate v3	Phosph ate v1
Collagen	1640 cm^{-1}	1545 cm^{-1}	1404 cm^{-1}	1236 cm^{-1}	1081 cm^{-1}	1031 cm^{-1}	-
Coll/MBG_SG_Sr4% /nano-HA	1637 cm^{-1}	1551 cm^{-1}	1411 cm^{-1}	1238 cm^{-1}	1089 cm^{-1}	1027 cm^{-1}	963 cm^{-1}
GEN/EtOH crosslinked Coll/MBG_SG_Sr4% /nano-HA	1652 cm^{-1}	1557 cm^{-1}	1410 cm^{-1}	1235 cm^{-1}	1090 cm^{-1}	1029 cm^{-1}	963 cm^{-1}

Considering the presence of MBG_SG_Sr4% particles in the system, the potential ion loss during and after the chemical treatment was assessed by ICP analysis. The percentage values reported in Figure 3.58 and Table 3.38 was calculated by considering the amount of strontium initially incorporated into MBG_SG_Sr4% particles (as measured by ICP on acid-digested samples) and the

content of the inorganic phase in the hybrid formulation, assuming the homogeneous distribution of the particles.

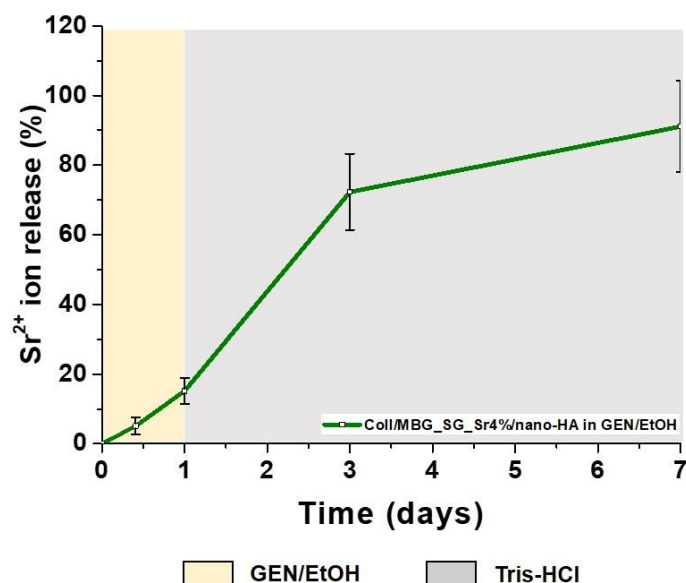


Figure 3. 58 Sr²⁺ ion release from GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA system.

According to the results obtained for the Coll/MBG_SG_Sr4% system, the chemical crosslinking performed in GEN/EtOH solution led to a limited release of ions, registering about 15% of ion loss at 24 hours. The samples were consequently transferred in Tris-HCl buffer up to 7 days. The release kinetics proved the complete loss of the incorporated strontium after 7 days of incubation, as observed for the other system.

Table 3. 38 Sr²⁺ release values (%) from GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA system.

	10h	24h	3d	7d
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	5.1% ± 2.4	15.1% ± 3.7	72.3% ± 10.9	91.1% ± 13.1

The effects of the genipin crosslinking over the visco-elastic properties of the Coll/MBG_SG_Sr4%/nano-HA system were detected by means of rheological tests. The potential increase of the material strength after the chemical treatment was investigated by means of a dynamic amplitude sweep test (0.01 – 1% strain; 1 Hz) while the temperature ramp up to 80 °C was performed to define the thermal stability of the system.

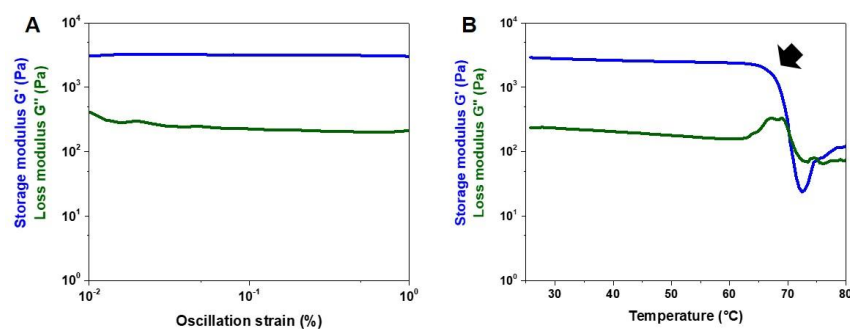


Figure 3. 59 Amplitude sweep tests (A) and temperature ramp (B) performed on GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA samples.

As reported in Figure 3.59A and Table 3.39, G' and G'' values of genipin crosslinked Coll/MBG_SG_Sr4% samples were registered to be about 3100 Pa and 216 Pa, respectively. The effective crosslinking was thus proved by an increase of almost ten times of the storage modulus of the system as well as the denaturation temperature. In fact, Figure 3.59B showed the significant drop of G' and G'' at about 65 °C, related to the denaturation of the collagen molecules and the subsequent collapse of the material structure.

Table 3. 39 Mean values of storage modulus (G'), loss modulus (G'') and denaturation temperature of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA.

	Storage modulus (G')	Loss modulus (G'')	Denaturation Temperature
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	3100 ± 71.8 Pa	216.2 ± 45.8 Pa	65 ± 1.2 °C

The stability of genipin crosslinked Coll/MBG_SG_Sr4%/nano-HA was further assessed by observing the degradation of material samples in collagenase and PBS solutions. In particular, Figure 3.60A showed the percentage of weight loss due to the enzymatic degradation of the material immersed in collagenase up to 48 hours, while Figure 3.60B represented the hydrolytic degradation of the samples in PBS up to 14 days.

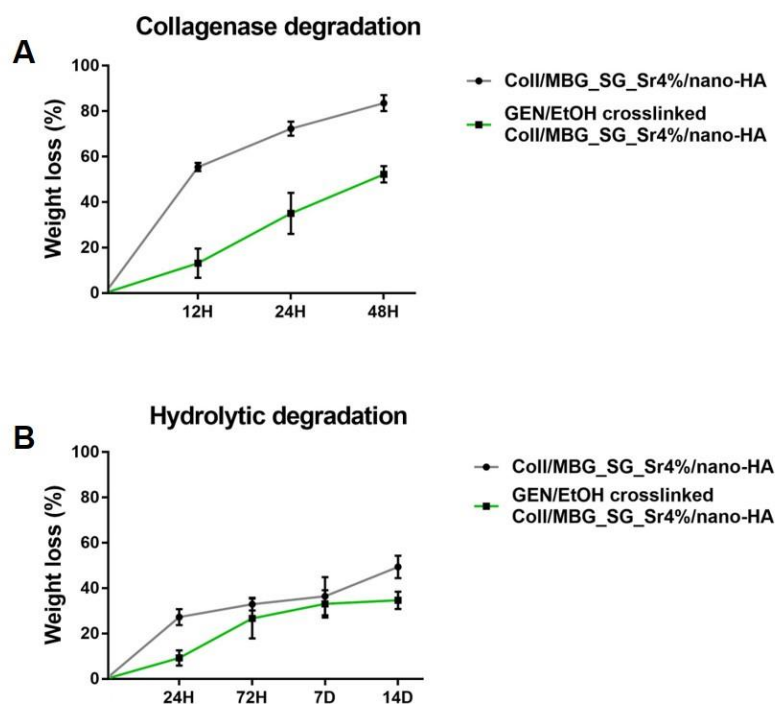


Figure 3.60 Enzymatic (A) and hydrolytic (B) degradation of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA.

The enzymatic degradation in collagenase confirmed the successful chemical crosslinking of the system, showing lower digestion kinetics associated to minor weight loss compared to the non-crosslinked system. In detail, the non-crosslinked samples registered more than 50% weight loss already after 12 hours of incubation, whereas a similar value was registered after 48 hours for the crosslinked samples, as reported in Table 3.40. Non-crosslinked samples lost more than 80% of the initial weight after 48 hours suggesting a complete digestion of the collagenous matrix, where the remaining weight can be associated to the non-digested inorganic phase.

After GEN/EtOH crosslinking, the covalent bonds formed between collagen molecules increased the resistance of the system subjected to the collagenase action.

Table 3.40 Weight loss (%) of GEN/EtOH crosslinked and non-crosslinked Coll/MBG_SG_Sr4%/nano-HA samples following incubation in collagenase up to 48 h.

	Weight loss at 12h	Weight loss at 24h	Weight loss at 48h
Coll/MBG_SG_Sr4%/nano-HA	55.4 ± 1.8	72.3 ± 3.1	83.5 ± 3.5
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	13.1 ± 6.4	35 ± 9.1	52.2 ± 3.6

Accordingly, the hydrolytic degradation showed an overall good stability of both crosslinked and non-crosslinked samples in saline solutions up to 14 days. However, genipin treated samples presented lower degradation kinetics, retaining

up to 66% of the initial weight at 14 days of incubation, while the non-crosslinked system lost almost half of the initial weight.

Table 3. 41 Weight loss (%) of GEN/EtOH crosslinked and non-crosslinked Coll/MBG_SG_Sr4%/nano-HA samples following hydrolytic degradation up to 14 days.

	Weight loss at 24h	Weight loss at 72h	Weight loss at 7d	Weight loss at 14d
Coll/MBG_SG_Sr4%/nano-HA	27.2 ± 3.5	32.9 ± 2.8	36.4 ± 8.4	49.3 ± 4.9
GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA	9.2 ± 3.3	26.6 ± 8.8	33 ± 6.1	34.6 ± 3.8

As previously observed for the Coll/MBG_SG_Sr4% and Coll/nano-HA systems, genipin induced a significant increase of the overall mechanical properties and stability of the collagen-based composite. In addition, the use of ethanol as solvent for the chemical reaction hindered the massive loss of strontium ions while enabling a better modulation of the release kinetics.

The presented results thus led to the selection of the GEN/EtOH solution for the stabilisation of 3D printed scaffolds derived from the processing of the developed collagen-based systems. According to these observations, the cytocompatibility of the crosslinking strategy was explored, as will be reported in Chapter 5.

3.6 Final considerations

The results reported in this Chapter proved the successful development of homogenous hybrid formulations based on the combination of type I collagen with strontium containing mesoporous bioactive glasses (Sr-MBGs) and rod-like nano-sized hydroxyapatite (nano-HA) particles.

The use of higher collagen concentrations as well as nano-sized Sr-MBGs (MBG_SG_Sr4%) showed higher advantages in the design of biomimetic nanocomposite materials compared to lower collagen content and the use of micro-sized Sr-MBGs. These conclusions derived from the greater bioactive character, the higher visco-elastic properties, and the biomimetic distribution of nanoparticles alongside the reconstituted collagen fibrils shown by the Coll/MBG_SG_Sr4% system.

The combination of nano-HA with type I collagen led to the development of a second formulation after the proper optimisation of the concentration of the dispersing agent (Darvan 821-A) exploited to hinder the formation of large HA agglomerates. The reconstituted composite material also proved higher stiffness compared to the MBG-containing system thanks to the greater interactions between hydroxyapatite and collagen molecules.

A third hybrid formulation was developed combining both MBG_SG_Sr4% and nano-HA particles to improve the final properties of the composite, creating a biomimetic and bioactive system for the design of 3D printed bone scaffolds.

A comprehensive rheological study was conducted on all the designed formulations, proving their suitability as biomaterial inks particularly due to their shear-thinning behaviour. However, the low shear recovery presented by the collagen-based materials suggested the use of supporting materials during the printing process.

Sr-MBG containing systems demonstrated their ability of releasing pro-osteogenic strontium ions, where the presence of the collagenous matrix enabled a better modulation of the ion loss up to 7 days, showing lower release kinetics compared to the MBG particles alone.

Furthermore, different chemical crosslinking methods were tested to increase the mechanical properties and the stability of the collagen-based composites. As a matter of fact, higher stiffness of the matrix is considered to provide better 3D conditions for osteogenic differentiation [6,9,60].

In contrast to the poor influence shown by both EDC/NHS and 4-StarPEG crosslinking over the final properties of the materials, the genipin treatment led to a significant increase of the material strength and stability for all the developed systems, detected by rheological and *in vitro* degradation tests. The superior crosslinking efficiency of genipin was attributed to the effective formation of stable covalent bonds between collagen molecules, according to previous studies [32]. Despite the enhancement of the mechanical properties and the stability was evident for all the systems, different stiffness values as well as degradation kinetics were observed according to the inorganic phase incorporated in the composite material.

Moreover, the use of ethanol as medium for the chemical crosslinking treatment hindered the massive loss of ions during cross-linking compared to the aqueous saline solutions. Considering that the chemical crosslinking represents the last step of the scaffold manufacturing process, it is fundamental to minimize the ion loss, guaranteeing almost the total release once the construct is incubated with cells.

Based on the previous results and considerations, Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA systems were used to design 3D printed scaffolds, while GEN/EtOH was selected as chemical crosslinking agent for the further stabilisation of the constructs, as described in Chapter 4.

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CHAPTER 4

Strategies for the extrusion 3D printing of collagen-based formulations: Results and Discussion

4.1 Introduction

Extrusion 3D printing technologies were selected to design collagen-based biomimetic scaffolds by processing the developed hybrid formulations, as described in the previous Chapter. In contrast to other techniques such as laser induced or inkjet printing, extrusion-based systems enable the processing of biomaterial inks with different physico-chemical features in a wider range of viscosities. However, despite the higher flexibility, this technique is characterised by lower resolution and precision of the final designed constructs [1,2].

In this frame, biomaterial printability and high printing fidelity are directly related to the specific viscoelastic properties of the processed materials, with clear requirements to allow for effective extrusion and deposition and eventually for successful optimization of the designed constructs [1]. These aspects are a critical issue when natural polymers are considered, as they must be treated in a narrow range of thermal and chemical conditions, and they normally require a support to avoid structure collapse and deformation while printing [3,4]. As a matter of fact, the direct printing of soft biomaterials still represents a challenge in this field, where the achievement of high printing fidelity and complex structures is strongly limited by the difficulties in supporting these materials during the manufacturing process.

In this chapter, the assessment of different strategies to obtain high resolution biomimetic scaffolds based on the developed collagen-based formulations and by means of extrusion 3D printing technologies will be described.

According to their rheological and physico-chemical properties, Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA hybrid formulations were printed exploiting the freeform reversible embedding of suspended hydrogels (FRESH) in order to support their deposition and avoiding the structure collapse [3]. Based on this, Section 4.2 will report the printability of the developed collagen-based suspensions, assessed using a commercial BIOX 3D Bioprinter (Cellink, Gothenburg, Sweden) and extruding the suspensions into a gelatine-based supporting bath (FRESH V1).

The visual inspection of the printed structures enabled the optimisation of the printing parameters for each suspension, while different methods to limit the presence of air bubbles in the materials were explored.

To achieve higher printing fidelity, a second gelatine-based slurry (FRESH V2) with enhanced morphological and rheological features was investigated to produce constructs with higher resolution according to the studies of Lee and co-workers [5].

The printed scaffolds were further chemically crosslinked using GEN/EtOH solutions, exploiting the strategy optimised and described in Section 3.5.3, in order to improve the stability of the final constructs.

A second strategy based on the UV curing of the deposited material during the printing process was explored and described in Section 4.3. To this aim, hybrid formulations were obtained starting from the protocols previously optimised and combining methacrylated type I collagen with MBG_SG_Sr4% and nano-HA particles. A cytocompatible photo-initiator (Irgacure 2959) was mixed to the material formulation to induce the crosslinking of the material upon UV exposure (365 nm).

A comprehensive rheological study was conducted to explore the UV curing kinetics and the visco-elastic properties of the materials, also using different concentrations of photo-initiator. Based on the rheological analysis, the printing process was optimised in order to enable the UV curing of the biomaterial inks layer by layer.

4.2 3D Printing of scaffolds exploiting the freeform reversible embedding of suspended hydrogels (FRESH)

Part of the work described in this chapter has been previously published [6,7].

This section will describe the strategies explored for the 3D extrusion printing of the developed collagen-based systems (Coll/MBG_SG_Sr4%, Coll/nano-HA, Coll/MBG_SG_Sr4%/nano-HA), based on the technique reported as freeform reverse embedding of suspended hydrogels (FRESH) [3,8].

With the aim to design and fabricate scaffolds with advanced biomimetic properties, 3D printing technologies can be exploited to realize more complex microarchitectures, enabling higher control over the final shape and architecture compared to the conventional manufacturing techniques [3,9,10]. The introduction of the extrusion-based 3D printing in bone tissue engineering has increased the precision and the repeatability of scaffold manufacturing, in addition to the overall process biocompatibility [3,9,11]. However, these techniques require specific rheological properties of the biomaterial inks such as shear thinning, yield stress and fast shear recovery in order to ensure printability and high printing fidelity [2,9,11]. In particular, the shear thinning behaviour is normally required to enable

the extrusion of the biomaterial inks especially through very thin needles associated to high shear stresses, while the transition to a solid state may be exploited to stabilise the extruded structure [1,3].

The rheological properties detected for all the developed hybrid formulations were thus considered as guiding reference to optimise the fabrication of 3D structures by extrusion-based printing process. Despite all the collagenous systems successfully proved a sharp decrease of the viscosity values upon increasing shear rates, the slow shear recovery and gelation kinetics as well as the low values of yield stress were considered limiting factors for the realisation of stable 3D constructs.

According to the previous observations and based on previous studies in the literature, to increase the final printing fidelity while avoiding the printed structure collapse due to the insufficient values of viscosity and yield stress in stationary conditions, the freeform reversible embedding of suspended hydrogels (FRESH method) was exploited [3,8]. This innovative technique exploits a biocompatible supporting bath to enable the deposition of complex biological structures in order to improve the printing fidelity. Once the structure is printed, the supporting material is removed, enabling the post processing of the printed construct.

In the case of collagenous inks, a gelatine-based bath can be used to allow the simultaneous gelation of the 3D structure and the melting of the supporting material by simply raising the temperature to 37 °C after printing [3].

In this study, the use of two different gelatine slurries (FRESH V1, FRESH V2) and their influence over the final properties of the scaffolds were explored. The printing parameters were optimised for each hybrid formulation in order to obtain high resolution and high shape fidelity according to the original 3D model. To this aim, mesh-like frameworks were selected to easily allow the evaluation of shape fidelity of the 3D-printed constructs, providing evidence of the system printability.

Finally, the optimised chemical crosslinking strategy based on the use of GEN/EtOH solutions, as described in Section 3.5.3, was exploited to improve the stability and the stiffness of the designed scaffolds.

4.2.1 Printing of scaffolds exploiting the gelatine slurry FRESH V1

4.2.1.1 Preparation and characterisation of the gelatine slurry V1

The use of a gelatine slurry to support the printing of scaffolds starting from soft biomaterials inks was first described by Hinton et al. [3] who observed that this supporting material was able to behave as a rigid body at zero-shear conditions and as a viscous fluid at high shear stresses, enabling the extrusion of soft inks and preserving the shape of the deposited filaments.

Following the procedures reported in the literature [3,8], the gelatine slurry, named as FRESH V1, was obtained as described in Section 2.2.12 and subsequently placed in the specific container to support the printing of the designed collagen-based inks.

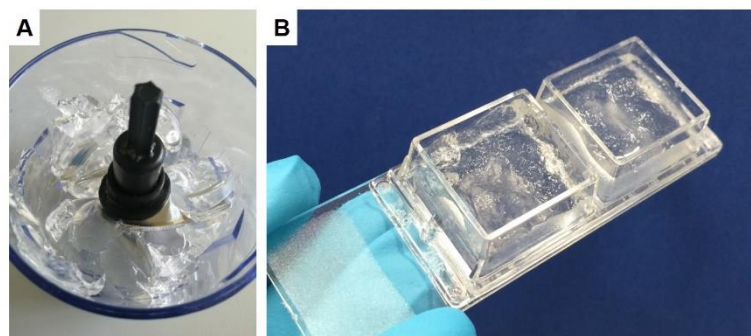


Figure 4. 1 Images showing the blending of the gelatine gel (A) and the final gelatine slurry FRESH VI (B).

As shown in Figure 4.1, the gelatine slurry was obtained after the blending and the post processing of a stiff gel of gelatine to produce a final microparticle suspension in PBS. After blending of the gel for 120 seconds (Figure 4.1A), subsequent and multiple centrifugation steps were exploited to remove dissolved gelatine and foam. After processing, the obtained gelatine particles were resuspended in pre-chilled PBS and stored at 4 °C to preserve their physico-chemical properties. Immediately before use, the slurry was centrifuged and poured into the printing container, regulating the volume according to the 3D model dimensions, always obtaining the supporting bath at least 2 mm higher than the scaffold thickness.

Before its use the resulting gelatine slurry FRESH V1 was analysed by means of rheological tests to determine its visco-elastic properties at 20 °C as well as its thermo-responsive behaviour, as shown in Figure 4.2.

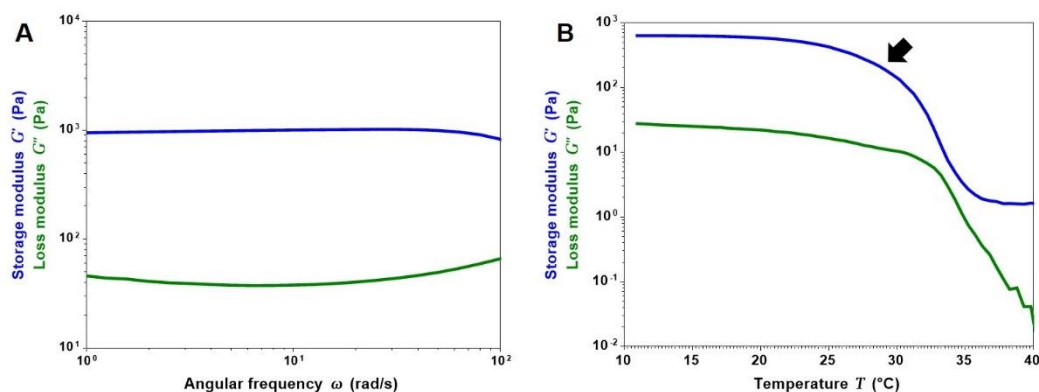


Figure 4. 2 . Storage and loss moduli (A) and gel to sol transition (B) of the gelatine slurry FRESH VI detected by means of frequency sweep and temperature ramp tests.

According to the results reported by Hinton and co-workers [3], the gelatine slurry proved a good stability at 20 °C, registering constant visco-elastic properties in a wide range of frequencies between 1 and 100 rad/s, characterised by values of storage modulus (G') and loss modulus (G'') of about 980 Pa and 43 Pa respectively (Table 4.1). Moreover, a visible drop of G' and G'' was observed at about 28 °C, proving the melting of the gelatine slurry FRESH V1 at increasing temperatures.

Table 4. 1 Visco-elastic properties at 20 °C and melting temperature of the gelatine slurry FRESH V1.

	Storage modulus (G')	Loss modulus (G'')	Melting Temperature
Gelatine slurry FRESH V1	980.7 ± 30.4 Pa	43.2 ± 5.9 Pa	28 ± 0.8 °C

The reported results thus proved that the gelatine slurry FRESH V1 is a stable supporting material at 20 °C and can be simply removed at temperatures higher than 30 °C due to the gel to sol transition.

4.2.1.2 Printability assessment and printing set-up

The printability of Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA systems was assessed using a temperature-controlled extrusion-based system and equipped with 27 G and 30 G metal needles, characterised by a length of 6.35 mm and an internal diameter of about 200 µm and 120 µm respectively, to potentially enhance the resolution of the final 3D constructs.

In detail, a temperature controlled pneumatic printhead was used to enable the material extrusion driven by regulating the air-pressure on the cartridge and keep a constant temperature of 10 °C in order to preserve the visco-elastic properties of the collagen-based suspensions. Moreover, as shown in Figure 4.3, 2-well chamber-slide containers were filled with the gelatine slurry FRESH V1 and placed on the printing-bed kept at 20 °C to avoid the material melting. Considering the overall dimensions of the temperature controlled printhead and the short length of the needles, 2-well chamber-slides were used to enable the correct calibration of the system while avoiding the crash between the different components.

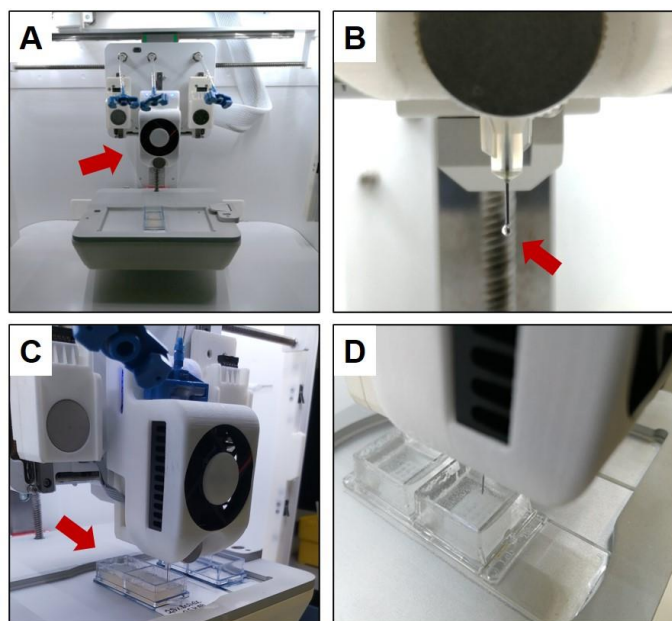


Figure 4.3 Printing set up using FRESH method: the BIOX bioprinter was equipped with a temperature-controlled pneumatic print-head (A) to enable the extrusion of the collagenous suspensions (B) into the gelatine slurry within 2-wells chamber-slides (B, C).

Despite the advantage of reaching enhanced resolution and precision of the designed constructs, the use of needles with smaller diameters is often reported to hinder the proper extrusion of the materials due to the high shear stresses involved [1,12]. The printability of materials with small size extruders is thus strongly related to their visco-elastic properties and shear-thinning behaviour [4]. In addition, the potential needle clogging due to the presence of air bubbles or material aggregates is even enhanced when using smaller diameter and higher length of the needle. For these reasons, most of the printing studies found in the literature report the use of extruders with diameters higher than 400 μm in size [8,13–17].

According to the previous observations, the printability of the developed Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA suspensions was firstly assessed using both 27 G and 30 G needle, observing the effective extrusion of the material upon application of pressures varying between 10 kPa and 100 kPa (Figure 4.3B). For the tests, the suspensions were centrifuged at 3000 rpm for 5 minutes to remove air bubbles, loaded into a 3 mL cartridge after tightening the selected needle, and subsequently mounted on the pneumatic print-head pre-conditioned at 10 °C.

This preliminary assessment showed that all the collagen-based suspensions were successfully extruded through both 27 and 30 G needles applying pressures between 60 and 90 kPa, where higher pressures were required when using smaller needles. These results are in line with the significant low values of viscosity (0.1-0.2 Pa.s) registered at the highest shear rate (10^3 s^{-1}) during the rheological analysis for all the hybrid formulations (Chapter 3). However, the continuous stop of the material flow particularly using 30 G needles, suggested the potential abundant presence of air in the material and mostly in the case of MBG_SG_Sr4% containing systems. This aspect can be related to the morphological features of Sr-MBG

particles characterised by high surface area and pore volume, leading to the subsequent greater incorporation of air in the system.

To further prove the printability of the designed collagenous materials while testing the supporting efficacy of the gelatine slurry FRESH V1, mesh-like structures ($10 \times 10 \times 1 \text{ mm}^3$) with infill density ranging between 15% and 30% were selected to easily allow the evaluation of shape fidelity and the deposition of material strands with the different needles.

Preliminary printing tests were performed using the Coll/MBG_SG_Sr4% system, where the suspension kept at 10 °C was extruded into the FRESH V1 supporting bath at 20 °C after setting the printing parameters using the integrated software (heartos_v1.7.0+165-dev) interface present on the printer.

As summarised in Table 4.2, pressures between 60-100 kPa were combined with printhead speeds of 7-8 mm/s while the z step varied in the range of 90-140 μm according to the size of the needle used.

After printing, the resulting scaffolds were incubated at 37 °C for 3 hours to promote the sol-gel transition of the Coll/MBG_SG_Sr4% system while removing the liquid gelatine. Subsequently, the resulting structures were visually inspected, as shown in Figure 4.4.

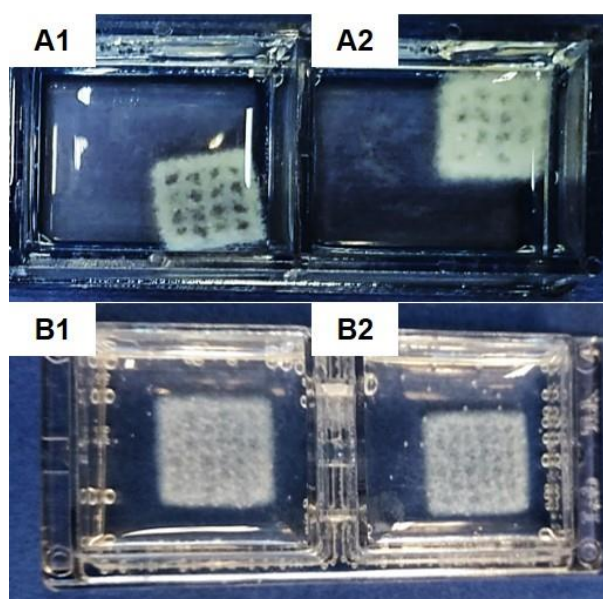


Figure 4. 4 Preliminary mesh-like scaffolds obtained by the extrusion printing of Coll/MBG_SG_Sr4% using 27 G and 30 G needles.

During the printing process, the rheological behaviour exhibited by the gelatine slurry at 20 °C enabled the accurate moving of the needle due to the negligible mechanical resistance while holding in place the deposited material. However, as clearly visible in Figure 4.4, the resulting constructs exhibited low definition and a hazy and “blotchy” aspect regardless the needle size. The suspension thus showed poor printability due to their inability to form uniform strands and retain distinct layers when stacked. Even after the incubation at 37 °C, the scaffolds reconstituted in a weak matrix that evidenced the presence of a significant amount of air, that led to the subsequent structure damage after the removal of the supporting material.

These observations can be connected with the repetitive clogging of the needle observed during the extrusion process due to the presence of small air bubbles detected at the needle entrance and to the resulting need of frequent interruption of the scaffold printing.

Table 4. 2 Parameters used for the preliminary printability assessment of the Coll/MBG_SG_Sr4% suspension.

	Needle	Pressure	Printhead speed	Z layer	Infill (%)
A1	27 G (200 μ m)	60 kPa	8 mm/s	140 μ m	15%
A2	27 G (200 μ m)	70 kPa	8 mm/s	140 μ m	15%
B1	30 G (120 μ m)	90 kPa	7 mm/s	90 μ m	30%
B2	30 G (120 μ m)	90 kPa	8 mm/s	90 μ m	30%

Despite rarely reported in the literature, the presence of air is considered one of the main concerns for the material printability and is often caused by the multiple stirring and mixing steps required to homogenize complex formulations [16]. High viscosity values as well as the presence of different phases in suspension further hamper the removal of air bubbles from the systems, and this can compromise the shape fidelity as well as the stability of the resulting scaffolds.

To solve this issue, different strategies were studied and explored to limit the presence of air in the developed collagen-based systems to obtain a constant flow of the material and better printing results. In detail, to promote the release of air while avoiding the sedimentation of the inorganic particles, all the suspensions were stored up to 24 hours in a vacuum chamber kept at 4 °C and subsequently centrifuged at 3000 rpm before printing. However, no evident improvements were obtained during the extrusion of the suspensions, as the presence of abundant air was still observed.

Consequently, a second procedure was optimised exploiting the high shear thinning behaviour showed by the collagen-based materials, able to reach very low viscosities at higher shear rates. According to this, the suspensions were kept stirring for about 1 hour at 4 °C and subsequently poured in 6 mL syringes, detecting an evident decrease of their viscosity due to the long stirring. Subsequently, two centrifugation steps were carried out to promote the release of air, facilitated by the lower material viscosity.

The developed strategy successfully proved a more constant material flow at lower pressures, where suspensions were successfully printed with both 27 G and 30 G needles.

4.2.1.3 Optimisation of the printing parameters for Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA suspensions

The reduction of air into the material formulations enabled an effective optimization of the process parameters and the subsequent fabrication of more consistent 3D printed structures.

As previously reported, 3D digital models for square scaffolds (10 x 10 mm surface) and up to 5 mm in thickness were selected from the .STL files library provided by the bioprinter manufacturer. A grid pattern and different percentages of infill (15%, 30%) were configured to obtain 3D structures showing pores of about 2.2 mm and 1.5 mm, respectively.

The optimisation of the extrusion parameters for all the suspensions was obtained varying print-head speed (7 - 15 mm/s), air pressure (20 - 100 kPa) and layer height (100 – 200 μ m) according to the specific biomaterial ink and the resulting accuracy of the filament extruded using 27 G and 30 G needles. As a matter of fact, the diameter of the extruded filaments and, consequently, the final resolution obtained are strictly related to the needle diameter selected, as well as the selected print-head speed and the imposed flow rates, which in turn depend on pressure and material rheological properties [1,4].

As discussed in the study of Paxton and co-workers [1], to obtain a filament size equal to that measured by the inner needle diameter, the pressure imparted by the printing system needs to be finely regulated to harmonize the flow rate with the printing head speed. Moreover, the z step is often regulated to range between 60% and 70% of the inner needle diameter to favour the adhesion of layers while avoiding their collapse.

The assessment of the printing parameters and the resulting structures obtained by the extrusion printing of the Coll/MBG_SG_Sr4% suspension into the gelatine slurry FRESH V1 were thus reported in Figure 4.5.

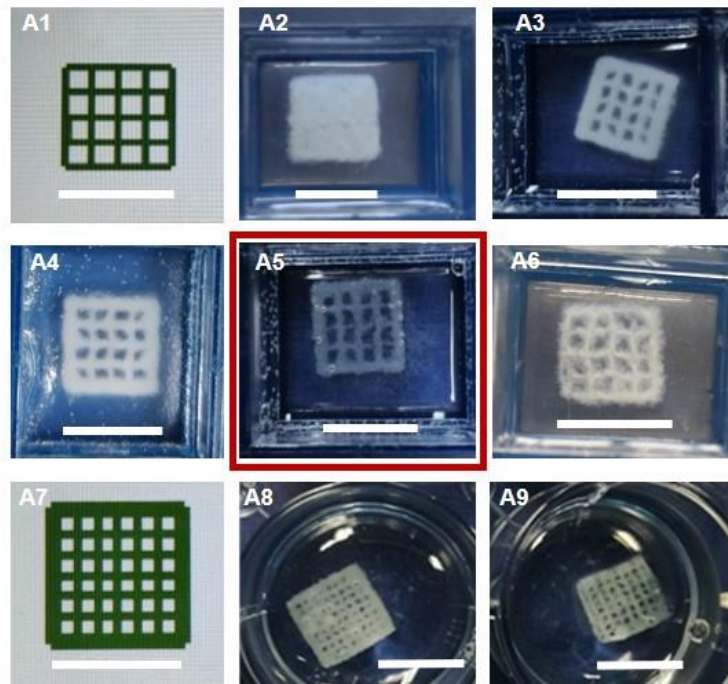


Figure 4.5 Mesh-like 3D printed scaffolds obtained by the extrusion printing of the Coll/MBG_SG_Sr4% suspension into the gelatine slurry FRESH V1 using different process parameters and needle size. The red square highlights the scaffold showing the best shape fidelity. Scale bar 10 mm.

The difference in fibre forming and stacking behaviour was assessed comparing the scaffolds obtained with different parameters and the geometrical features of CAD models (Figure 4.5 A1, Figure 4.5 A7). Despite droplet-like fibres were observed during the extrusion of the Coll/MBG_SG_Sr4% suspension (Figure 4.3B), coherent and continuous strands were deposited maintaining a 3D cross-pattern structure.

Reducing the extrusion pressure and printing speed significantly improved the printing resolution, which translated in clearer features and more defined strands, and pore geometry. Abundant material dispensing due to higher pressures led to the formation of bulky structures (Figure 4.5 A2) while higher speeds associated to lower material flow were responsible for thinner and warped constructs (Figure 4.5 A6). Moreover, increasing the layer height from 0.14 mm (Figure 4.5 A4) to 0.17 mm (Figure 4.5 A5) led to higher resolution, where greater z steps limited the excess of material and thus the enlargement of the deposited strand.

The scaffolds imaged in Figure 4.5 thus proved how the proper optimization of the printing parameters led to obtain structures closely resembling geometry and dimensions of the selected CAD files. However, a partial distortion of the structure was occasionally observed on the filament crossing, possibly due to the dragging and excess of material, as also reported in other studies [1].

As reported in Table 4.3, for the Coll/MBG_SG_Sr4% system, the best shape fidelity was obtained using a pressure of 30 kPa, a speed of 10 mm/s and a z step of 0.17 mm when using 27 G needles, while pressure and z step were varied to be 50 kPa and 0.10 mm for 30 G needles.

Table 4. 3 Printing parameters used for the design of scaffolds by the extrusion printing of the Coll/MBG_SG_Sr4% suspension into the gelatine slurry FRESH V1.

	Needle	Pressure	Printhead speed	Z layer	Infill (%)
A2	27 G (200 μ m)	50 kPa	10 mm/s	140 μ m	15%
A3	27 G (200 μ m)	40 kPa	10 mm/s	140 μ m	15%
A4	27 G (200 μ m)	30 kPa	10 mm/s	140 μ m	15%
A5	27 G (200 μ m)	30 kPa	10 mm/s	170 μ m	15%
A6	27 G (200 μ m)	30 kPa	12 mm/s	170 μ m	15%
A8	30 G (120 μ m)	50 kPa	10 mm/s	100 μ m	30%
A9	27 G (120 μ m)	50 kPa	10 mm/s	100 μ m	30%

Similar results were observed for the Coll/nano-HA suspension, where the optimization of the printing parameters led to the design of more defined scaffolds showing higher shape fidelity, as demonstrated in Figure 4.6.

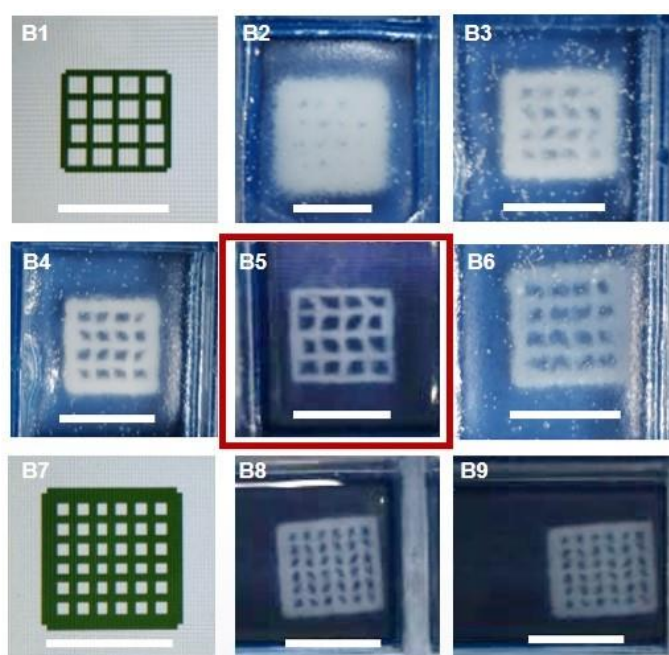


Figure 4. 6 Mesh-like 3D printed scaffolds obtained by the extrusion printing of the Coll/nano-HA suspension into the gelatine slurry FRESH V1 using different process parameters and needle size. The red square highlights the scaffold showing the best shape fidelity. Scale bar 10 mm.

According to the data reported in Table 4.4, when using 27 G needles, pressure and printhead speed were regulated to be 40 kPa and 11 mm/s, respectively, to enable the extrusion of filaments with size reproducing the inner needle diameter and arranged in mesh-like structures. Higher pressures and lower printhead speed led to the printing of bulky and less accurate structures.

In parallel, the best shape fidelity was obtained increasing the pressure up to 60 kPa and setting a z step of 0.10 mm by using 30 G needles.

As previously observed for the Coll/MBG_SG_Sr4% suspension, despite the good definition of the scaffolds, a partial deviation from the rectilinear pattern was evidenced in all the printed structures.

Table 4. 4 Printing parameters used for the design of scaffolds by the extrusion printing of the Coll/nano-HA suspension into the gelatine slurry FRESH V1.

	Needle	Pressure	Printhead speed	Z layer	Infill (%)
B2	27 G (200 μ m)	50 kPa	8 mm/s	140 μ m	15%
B3	27 G (200 μ m)	50 kPa	10 mm/s	140 μ m	15%
B4	27 G (200 μ m)	40 kPa	11 mm/s	140 μ m	15%
B5	27 G (200 μ m)	40 kPa	11 mm/s	170 μ m	15%
B6	27 G (200 μ m)	40 kPa	10 mm/s	170 μ m	15%
B8	30 G (120 μ m)	60 kPa	11 mm/s	100 μ m	30%
B9	27 G (120 μ m)	60 kPa	10 mm/s	100 μ m	30%

Finally, printing tests were performed with the Coll/MBG_SG_Sr4%/nano-HA suspension, as visible in Figure 4.7.

Based on the similar rheological properties exhibited by the three collagen-based systems, the optimal parameters identified for Coll/MBG_SG_Sr4%/nano-HA were consistent with those reported by the other two biomaterial inks.

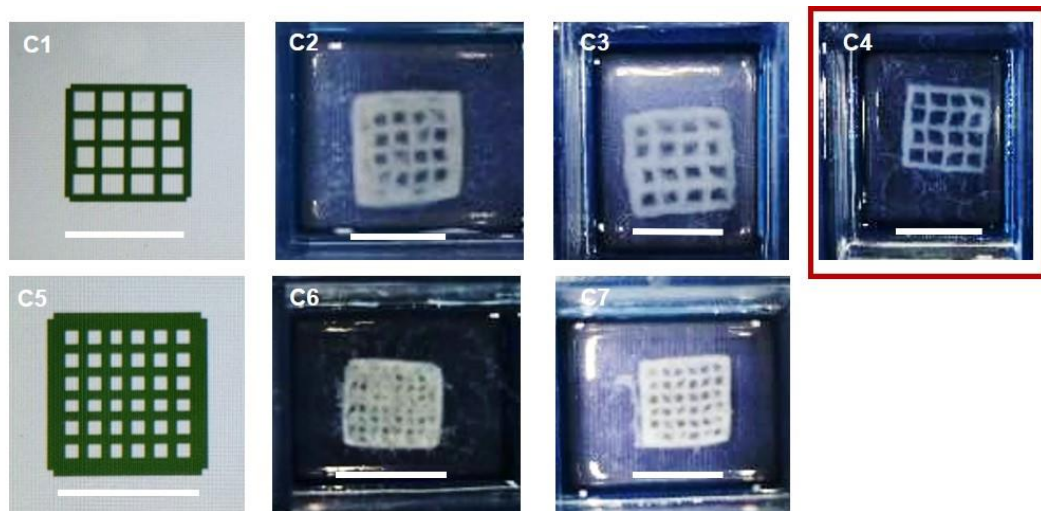


Figure 4. 7 Mesh-like 3D printed scaffolds obtained by the extrusion printing of the Coll/MBG_SG_Sr4%/nano-HA suspension into the gelatine slurry FRESH V1 using different process parameters and needle size. The red square highlights the scaffold showing the best shape fidelity. Scale bar 10 mm.

Accordingly, 30 kPa, 11 mm/s and 0.17 mm were set as optimized pressure, printing speed and z layer to achieve the best printing fidelity when using 27 G needles.

As previously observed, the use of thinner 30 G needles highlighted greater difficulties, and several times, the presence of residual air bubbles provoked the stop of the printing process and thus affected the scaffold manufacturing. However, good results in terms of shape fidelity were obtained setting a pressure of 50 kPa at 10 mm/s speed and 0.10 mm z layer.

Table 4. 5 Printing parameters used for the design of scaffolds by the extrusion printing of the Coll/MBG_SG_Sr4%/nano-HA suspension into the gelatine slurry FRESH V1.

	Needle	Pressure	Printhead speed	Z layer	Infill (%)
C2	27 G (200 μ m)	40 kPa	10 mm/s	140 μ m	15%
C3	27 G (200 μ m)	30 kPa	10 mm/s	170 μ m	15%
C4	27 G (200 μ m)	30 kPa	11 mm/s	170 μ m	15%
C6	30 G (120 μ m)	50 kPa	11 mm/s	100 μ m	30%
C7	30 G (120 μ m)	50 kPa	10 mm/s	100 μ m	30%

At this stage, the printed constructs were incubated at 37°C for 3 hours to liquefy and remove the gelatin extrusion bath while promoting the sol-gel transition of the printed material.

As visible in Figure 4.8, scaffolds maintained their original thickness up to 5 mm even after the removal of the supporting material and the washing step.

However, the soft nature of the reconstituted matrix after incubation at 37 °C evidenced the low stability of the scaffolds, often leading to their deformation or damage, thus hindering a more precise characterization. As a matter of fact, the present results suggested the need to improve the mechanical properties of the designed scaffolds in order to obtain more stable, complex and precise structures.

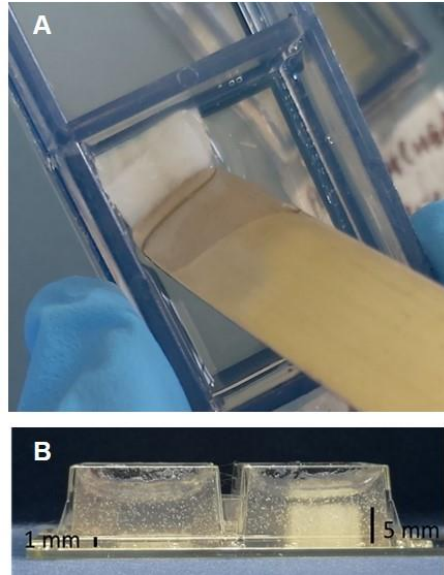


Figure 4. 8 Scaffolds obtained by the extrusion printing of the collagen-based suspensions into the gelatine slurry having 1 mm and 5 mm thickness, before (B) and after the removal of the material support (A).

Despite the collagen-based systems proved their printability with 30 G needles, the difficulties detected during the extrusion of the material due to the presence of residual air, support the selection of 27 G needles for the following printing tests.

4.2.2 Printing of scaffolds exploiting the gelatine slurry FRESH V2

Despite the positive viscoelastic properties and the good printability of the systems, the poor construct stability after removal of the gelatine bath and the observed structure imperfections suggested a further refinement of the printing process.

According to the studies recently conducted by Lee et al. [5], the use of an alternative gelatine slurry with improved morphological and rheological features was exploited to enhance the final resolution and printing fidelity of the scaffolds. Based on the observation of Lee and co-workers [5], this second gelatine slurry, named as FRESH V2, was produced in order to obtain uniform and spherical gelatine microparticles ranging between 20 and 30 μm , characterised by an optimised saline concentration and buffering capacity able to induce a faster self-assembly of collagen once extruded. On the contrary, the first version of the FRESH slurry (FRESH V1) was characterised by irregular shaped and larger particles that could partially hinder the correct deposition of the printed filaments.

As shown in Figure 4.9, the slurry FRESH V2 was obtained by rehydrating and resuspending the dried gelatine microparticles provided by Advanced Biomatrix

(LifeSupport, Advanced Biomatrix, Carlsbad, USA) in pre-chilled 10X PBS following the manufacturer's instructions.

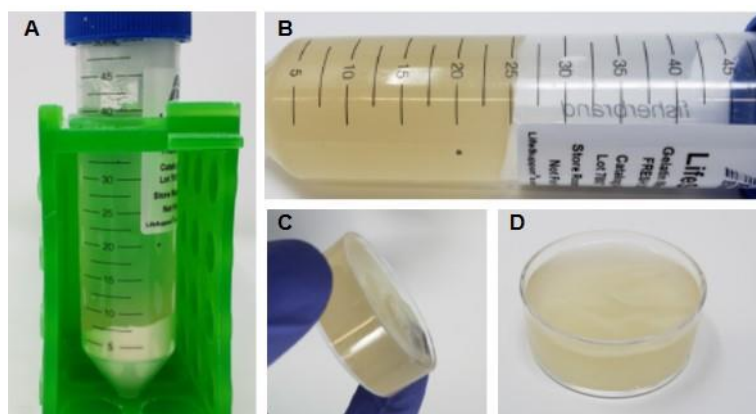


Figure 4. 9 Preparation of the gelatine slurry FRESH V2: dried powders (A); reconstituted gelatine slurry (B); petri dish filled with the gelatine slurry and showing its plastic behaviour (C, D).

Similarly to the previous gelatine bath, the gelatine slurry FRESH V2 showed an evident plastic behaviour at 20 °C, where no flow was observed even twisting the container.

Once prepared, the visco-elastic properties of the slurry at 20 °C and with increasing temperatures were studied by means of rheological tests as reported in Figure 4.10.

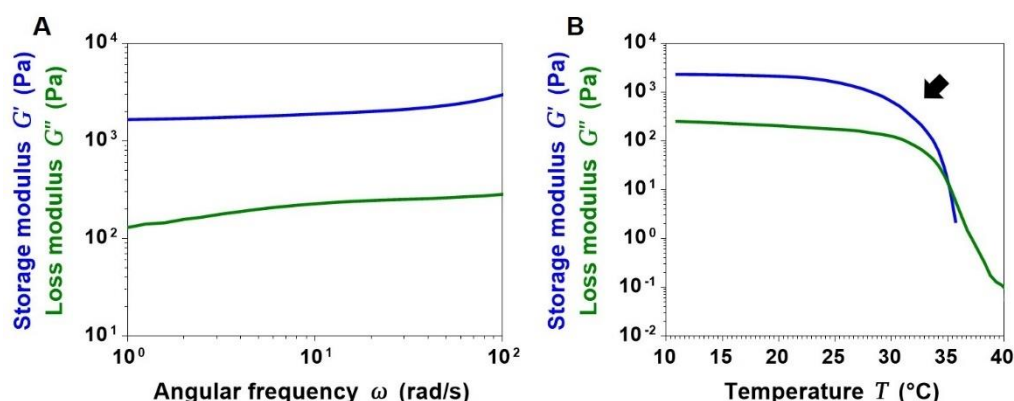


Figure 4. 10 Visco-elastic properties of the gelatine slurry FRESH V2 expressed as values of storage (G') and loss (G'') moduli in function of increasing frequencies (A) and increasing temperatures (B).

The stability and the stiffness of the supporting bath was explored by means of frequency sweep tests (Figure 4.10) that evidenced a solid-like behaviour in a wide range of frequencies ranging between 1 and 10^2 rad/s. The test highlighted also higher values of G' and G'' compared to the gelatine slurry FRESH V1, as summarised in Table 4.6.

The maintenance of the visco-elastic properties at room temperature was confirmed by means of the temperature ramp represented in Figure 4.10B, where the drop of G' and G'' was detected for temperatures higher than 30 °C. These results confirmed the melting of the gelatine slurry at temperatures that, in contrast, are known to promote the gelation of the collagen-based systems.

Table 4. 6 Visco-elastic properties at 20 °C and melting temperature of the gelatine slurry FRESH V2 and gelatine slurry FRESH V1.

	Storage modulus (G')	Loss modulus (G'')	Melting Temperature
Gelatine slurry V2	1959.1 ± 349.6 Pa	206.9 ± 52.4 Pa	30.0 ± 0.5 °C
Gelatine slurry V1	980.7 ± 30.4 Pa	43.2 ± 5.9 Pa	28.0 ± 0.8 °C

The gelatine slurry FRESH V2 was subsequently tested for the extrusion printing of scaffolds, exploiting the same printing set-up presented in the previous section. In brief, a temperature controlled pneumatic printhead kept at 10 °C was used to enable the extrusion of the suspension into the gelatine bath maintained at 20 °C, as shown in Figure 4.11.

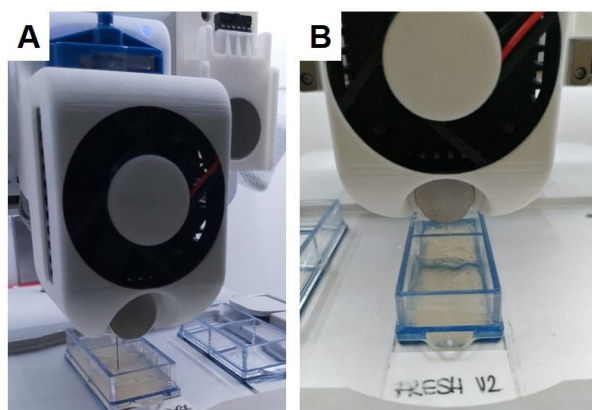


Figure 4. 11 Printing set up using FRESH method: the suspension was extruded by means of a temperature-controlled pneumatic print-head kept at 10 °C (A) into the gelatine slurry FRESH V2 (B).

The Coll/MBG_SG_Sr4%/nano-HA suspension was selected for the preliminary tests using the supporting bath FRESH V2, due to the high printing fidelity showed after the optimisation of the printing parameters while using the gelatine slurry FRESH V1. The Coll/MBG_SG_Sr4%/nano-HA suspension was then extruded into the FRESH V2 to fabricate scaffolds with a square surface (10 x 10 mm) with 15% infill density and up to 5 mm in thickness. Grid and honeycomb infill patterns were used to test the ability to reproduce different morphologies, as represented in Figure 4.12.

Based on the previous printability assessment, a pressure of 30 kPa associated to a printhead speed of 10 mm/s and a z step of 0.17 mm were set to obtain scaffolds with high shape fidelity.

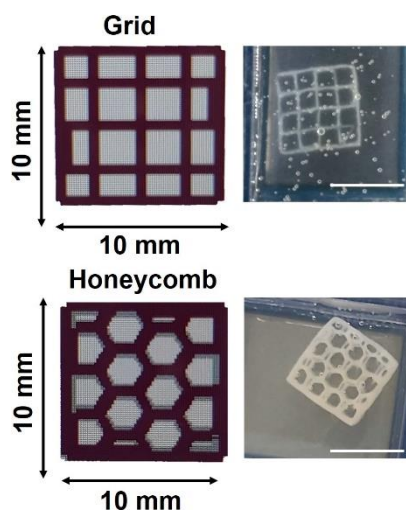


Figure 4.12 Mesh-like and honeycomb 3D printed scaffolds obtained by the extrusion printing of the Coll/MBG_SG_Sr4%/nano-HA suspension into the gelatine slurry FRESH V2 using 27 G needle. Scale bar 10 mm.

After printing, an incubation at 37 °C for 3 hours led to the sol-gel transition of the collagen-based system also supported by the optimised saline concentration of the bath, and the concurrent melting of gelatine.

The resulting scaffolds obtained using the gelatine slurry FRESH V2 and represented in Figure 4.12, proved high fidelity when compared to the selected CAD models. Both the grid and honeycomb patterns were precisely reproduced without observing evident distortion of the deposited filaments.

According to the results obtained by Lee et al. [5], the smaller size of the gelatine particles as well as their spherical morphology, promoted a better deposition of the material strands within the bath, minimising potential alteration of the printed structure.

Despite the better results obtained in terms of printing fidelity, the weak mechanical properties of the reconstituted material hindered the easy handling of the scaffolds thus requiring the use of crosslinking strategies to improve the final stability.

4.2.3 Chemical crosslinking of the 3D printed scaffolds

Based on the studies conducted about the chemical crosslinking of the collagen-based systems and described in Section 3.5.3, a 0.5 wt% genipin in 70% ethanol solution (GEN/EtOH) was exploited to promote the formation of covalent bonds between collagen molecules and the consequent increase of the material stability. As previously reported, the chemical treatment with GEN/EtOH led to a significant enhancement of the mechanical and thermal stability of the developed composites, allowing a more modulated release of strontium ions in the case of MBG_SG_Sr4% containing systems.

Scaffolds obtained by the extrusion printing of the developed collagen-based suspensions into the gelatine slurry FRESH V1 and FRESH V2 were thus

crosslinked by means of GEN/EtOH, exploring the effects of the chemical treatment over the final resolution and definition of the fabricated constructs.

After printing, the 3D scaffolds were incubated at 37 °C for 3 hours to induce the sol-gel transition of the collagenous materials while removing the supporting bath. The scaffolds were subsequently washed and immersed in GEN/EtOH solution, promoting the chemical crosslinking at 37 °C for 24 hours.

As visible in Figure 4.13, the visual inspection of the scaffolds after treatment with GEN/EtOH highlighted a partial loss of their definition, especially in the denser peripheral parts.

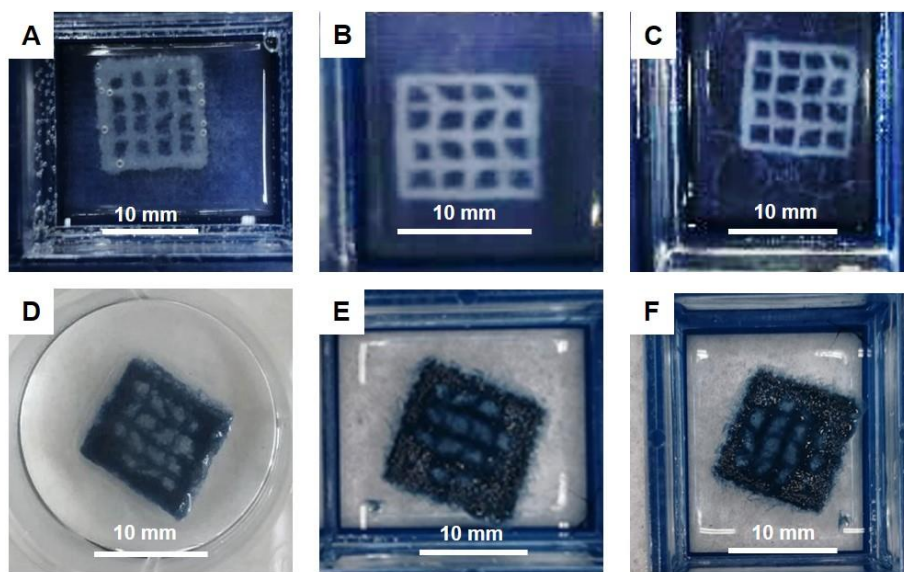


Figure 4. 13. Scaffolds of Coll/MBG_SG_Sr4% (A, D), Coll/nano-HA (B, E) and Coll/MBG_SG_Sr4%/nano-HA (C, F) before and after chemical crosslinking with GEN/EtOH

The thickening of the strands as well as the partial loss of the defined printed morphology can be attributed to the presence of residual gelatine particles of slurry FRESH V1. As reported by other studies, the irregular shaped and larger particles of the gelatine bath can easily get stuck into the collagenous structure, particularly in the corner and small pores [18]. Since genipin is also able to chemically crosslink gelatine [19], the residual supporting material can be crosslinked together with collagen molecules, leading to an alteration of the structural features as well as to a reduction of the final shape fidelity of the scaffolds.

Alongside the macro-scale properties of the final constructs, morphological analyses were conducted to observe the material micro and nano-architecture resulting after the printing process and the chemical crosslinking treatment.

Scaffolds of Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA realised by extrusion printing in FRESH V1 and subsequently crosslinked with GEN/EtOH, were collected, and lyophilised to perform FE-SEM analysis, as shown in Figure 4.14.

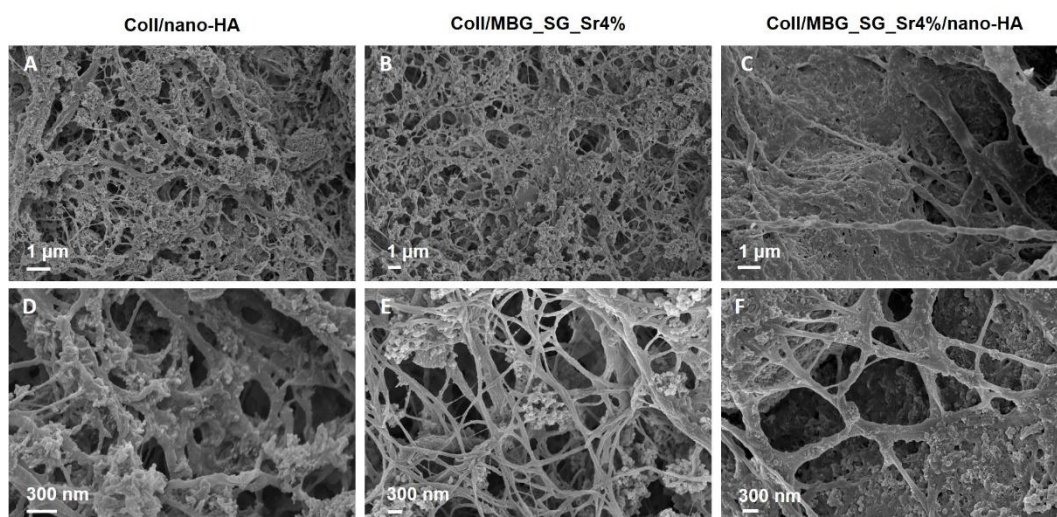


Figure 4. 14 Cross-sectional FE-SEM images of GEN/EtOH crosslinked scaffolds of Coll/nano-HA (A, D), Coll/MBG_SG_Sr4% (B, E) and Coll/MBG_SG_Sr4%/nano-HA (C, F) at different magnifications.

FE-SEM analysis confirmed the successful self-assembly of collagen and the subsequent formation of a highly fibrillar structure for all the scaffolds, as visible in Figure 4.14. The preparation of the biomaterial inks and the overall printing steps proved not to alter the homogeneous distribution of the inorganic phases into the collagenous matrix, where FE-SEM images at higher magnifications (Figure 4.14 D, E, F) also showed the distribution of MBG_SG_Sr4% and nano-HA nanoparticles alongside collagen fibres.

To prove the potential increase of scaffolds resolution even after the chemical crosslinking with genipin, the scaffolds produced by extrusion in FRESH V2 were morphologically analysed and compared to the structures obtained with FRESH V1.

Mesh-like and honeycomb scaffolds of Coll/MBG_SG_Sr4%/nano-HA realised by extrusion printing in FRESH V2 and subsequently crosslinked with GEN/EtOH, were collected, and visually inspected after washing, as shown in Figure 4.15.

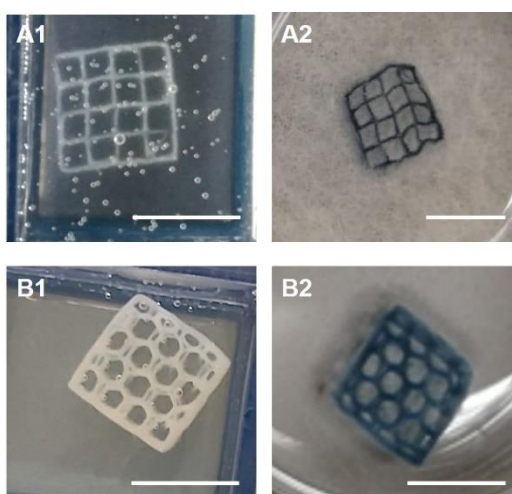


Figure 4. 15 Mesh-like and honeycomb scaffolds of Coll/MBG_SG_Sr4%/nano-HA before (A1, B1) and after (A2, B2) chemical crosslinking with GEN/EtOH. Scale bar 10 mm.

As clearly visible in Figure 4.15A2 and Figure 4.15B2, the scaffolds treated with genipin maintained the morphological features of the non-crosslinked printed structures (Figure 4.15 A1, B1).

According to the observation of Lee and co-workers [5], the smaller dimension and the spherical morphology of the gelatine particles favoured a more accurate washing of the bath, avoiding residuals between the pores of the crosslinked structures.

Moreover, the crosslinking treatment led to the overall increase of the scaffold stiffness enabling an easier handling and the further analysis of the constructs.

To better appreciate the final dimensions, the crosslinked scaffolds obtained using FRESH V1 and FRESH V2 were lyophilised to enable the morphological assessment by means of optical and scanning electron microscope (SEM), as reported in Figure 4.16.

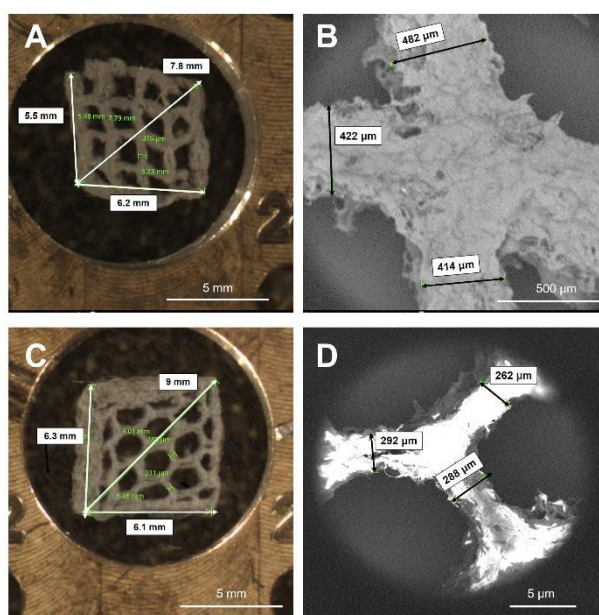


Figure 4. 16 Morphological assessment of chemical crosslinked lyophilised scaffolds obtained by means of extrusion in gelatine slurry FRESH V1 (A, B) and FRESH V2 (C, D) performed by means of optical microscope (A, C) and SEM (C, D).

Scaffolds obtained by the extrusion in FRESH V1 (Figure 4.16 A) and FRESH V2 (Figure 4.16C) both showed an overall slight shrinkage of the structure compared to the original dimensions and a partial distortion mainly due to the lyophilisation process and the chemical crosslinking treatment [20,21]. SEM images of the scaffolds (Figure 4.16B, D) enabled a more accurate dimensional analysis of the filaments, demonstrating a better shape fidelity and retention in scaffolds realised by means of FRESH V2, where the strand size was measured to be similar at the internal diameter of the needle used for the printing (about 200 μm) even after all the post processing steps.

According to the observations of Lee and co-workers [5], the gelatine particles of the slurry are moved to enable the material deposition during the printing process but can largely influence the final morphology of the filament. According to their studies, the smallest filament obtainable with FRESH V1 measured about 300 μm

in mean diameter, also observing not uniform morphology of the strands. On the contrary, in the same study, they claimed that the use of FRESH V2 led to the printing of filaments less than 100 μm in diameter, presenting highly repeatably and homogeneous morphologies. Moreover, their study also demonstrated a higher stability of the scaffolds obtained exploiting highly concentrated collagen solutions with acidic pH due to the buffering capacity of the bath FRESH V2 [3–5]. Therefore, this strategy is currently under investigation with the aim to increase the strength of the construct before removing the supporting material.

Based on the results presented, we proved the printability of the developed collagen-based systems and the enhanced shape fidelity obtained using gelatine slurry FRESH V2, thanks to its morphological and rheological features. In addition, the chemical crosslinking with GEN/EtOH showed its strengthening effect on the printed scaffolds, without significantly altering the final morphological properties.

However, the design of more complex structures was hindered by the soft nature of the collagen-based composites, observing the partial collapse of suspended parts upon the removal of the supporting material. Based on this, an alternative strategy to obtain a previous stabilisation of the material during the deposition step was explored and reported in the next section.

4.3 Preliminary assessment of the printability of UV curable collagen-based formulations

This section will describe the development, characterisation and preliminary printing assessment of hybrid formulations based on methacrylated collagen to obtain UV curable 3D printed scaffolds.

The use of photo-curable materials was explored to provide an initial stability of the 3D constructs while printing, considering the limitations presented by the developed collagen-based formulations in the design of complex architectures.

In this frame, methacrylated collagen has already been studied as effective UV curable material presenting enhanced matrix strength while preserving the physico-chemical features of native type I collagen [22–24]. However, few data are reported about the use of methacrylated collagen as UV curable system for 3D extrusion printing applications [25,26].

Printing accuracy could be improved by changing the physicochemical properties of the feed material, which in this case could be attained through the stiffening of the matrices while they are printed. In the attempt to print more complex structures, with a higher structural integrity, new hybrid formulations based on the use of methacrylated collagen were investigated. The idea was to replace natural type I bovine collagen with methacrylated type I bovine collagen (COLMA) in the material formulation. This could promote the crosslinking between collagen molecules upon application of UV light and in presence of a cytocompatible photoinitiator (Irgacure 2959), leading to improved mechanical properties upon deposition and consequently an improved accuracy of the printed features.

Therefore, the development of UV curable hybrid formulations based on methacrylated collagen and its combination with MBG_SG_Sr4% and nano-HA was carried out, observing the visco-elastic properties of the newly designed systems. Based on the rheological data, a preliminary assessment of the printing process exploiting the layer-by-layer UV curing of the scaffold was reported.

4.3.1 Design and characterisation of UV curable collagen-based systems

Type I methacrylated collagen (COLMA) was used to obtain hybrid formulations according to the supplier instructions (PhotoCol-IRG, Advanced Biomatrix) and based on the protocols optimised for the previously described collagen-based biomaterial inks.

For each suspension, the amount of collagen powders, inorganic phase and solvent has been calculated accordingly to the ratios reported in the PhotoCol-IRG instruction protocol, in order to properly balance the final concentration of solvent and neutralisation solutions. Moreover, the weight amount of each component was calculated respecting the ratio between organic and inorganic phase present in natural bone tissue (53 vol% of collagen and 47 vol% of the inorganic phase) [27] and achieving a final collagen concentration of 1.5 wt%. Accordingly, COLMA powders were dissolved in 20 mM acetic acid, while a mixture of Irgacure 2959 and neutralisation buffer (10X PBS) was subsequently added to neutralise the pH of the system while enabling the UV curing. As reported in Section 2.2.4, for the hybrid formulations, MBG_SG_Sr4% and nano-HA were firstly resuspended in acetic acid and neutralisation buffer respectively, before mixing into the COLMA solution.

The UV-curing capability of the material was firstly assessed exploring the effect on increasing concentrations of photoinitiator on the crosslinking kinetics.

The presence of Irgacure 2959 at 0.1 wt%, 0.5 wt% and 1 wt% in the final system was explored according to the values reported in previous studies, avoiding high concentrations to preserve the overall cytocompatibility of the material [26,28]. COLMA solutions containing 0.1, 0.5, and 1 wt% Irgacure 2959 were analysed by means of oscillatory rheological tests, keeping constant the frequency (1 Hz) and the amplitude (1% strain) of the stress applied, while subjected to UV light (356 nm, according to the photoinitiator used) at 15 mW/cm² for 1200 seconds.

The variation of their visco-elastic properties upon application of UV light was shown in Figure 4.17.

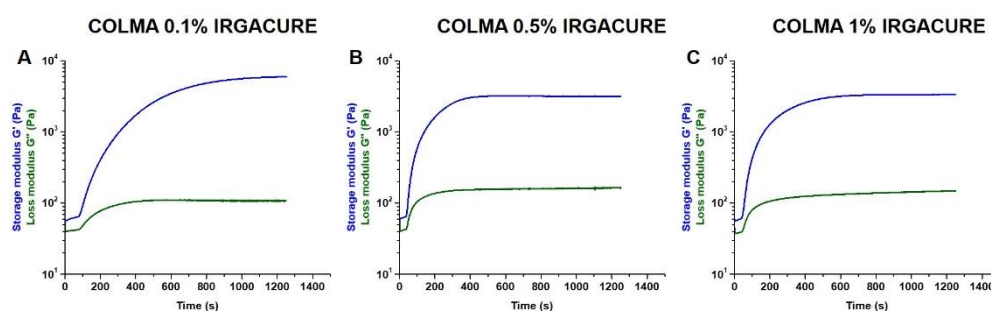


Figure 4. 17 UV curing kinetics of COLMA solutions containing different concentration of photoinitiator.

The curves represented in Figure 4.17 clearly proved the UV curing ability and kinetics of the COLMA solutions at different concentrations of photoinitiator, showing an evident and fast increase of the storage (G') modulus of the system when subjected to UV light.

As summarised in Table 4.7, higher concentrations of Irgacure 2959 led to faster curing kinetics, registering values of about 587 and 421 Pa of G' for the solution containing 0.5 wt% and 1 wt%, respectively, compared to only 91 Pa for the lowest concentration (0.1 wt%). After 1200 seconds of UV light application, G' and G'' reported similar values for all the tested systems, suggesting the achievement of the highest crosslinking efficacy while evidencing that greater concentrations of photoinitiator were not able to induce enhanced crosslinking of the material. However, faster curing kinetics are desirable considering the printing applications, favouring a more effecting crosslinking of the material layer by layer, also reducing the time of the process. According to these observations, the final Irgacure concentration for the systems was optimised to be 0.5 wt% in order to promote high crosslinking efficiency. Even if 0.5 wt% and 1 wt% Irgacure showed similar values of matrix stiffness and crosslinking kinetics, the lower concentration was selected to limit potential cytotoxic effects induced by the photoinitiator.

Table 4. 7 G' , G'' values and gel time of COLMA solutions containing different concentrations of Irgacure 2959.

	G', G'' after 100 s (UV 15 mW/cm ²)	G', G'' after 1200 s (UV 15 mW/cm ²)	Gel time
COLMA (0.1 wt% IRGACURE)	91.9 ± 10.1 Pa 48.1 ± 7.5 Pa	3931.4 ± 213.4 Pa 108.8 ± 23.6 Pa	< 200 s
COLMA (0.5 wt% IRGACURE)	587.6 ± 35.5 Pa 104.6 ± 22.1 Pa	3171.1 ± 199.8 Pa 163.4 ± 18.8 Pa	< 100 s
COLMA (1 wt% IRGACURE)	421.1 ± 33.7 Pa 79.7 ± 19.8 Pa	3351.6 ± 266.2 Pa 148.4 ± 32.4 Pa	< 100 s

The characterisation of hybrid formulations containing 0.5 wt% Irgacure was subsequently performed by means of a comprehensive rheological study, aimed at

exploring the printability of the COLMA-based systems as well as providing indications to further optimise the printing process.

To this aim, nano-HA (COLMA/nano-HA) and MBG_SG_Sr4% (COLMA/MBG_SG_Sr4%) containing suspensions were studied for their ability to be UV cured, over time, and compared with the COLMA solution. After loading onto the support plate, the material samples were subjected to UV light at 365 nm, 15 mW/cm² for 1200 seconds. While irradiating, the variation of the storage modulus (G') and the loss modulus (G'') was registered by applying an oscillating stress at 1% strain and 1 Hz. The variation of the viscoelastic properties was observed at room temperature and reported in Figure 4.18.

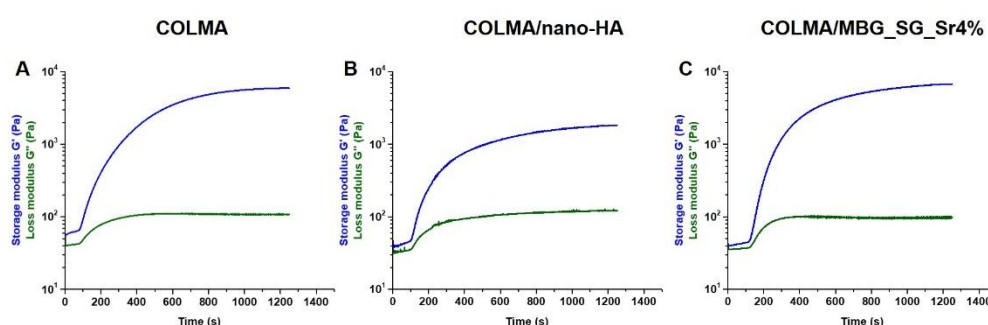


Figure 4. 18 UV curing kinetics of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems.

The UV curing tests performed on COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems containing 0.5 wt% Irgacure showed a significant increment of the viscoelastic properties of the materials upon UV light application. In fact, a sol-gel transition curve due to the UV curing of collagen was observed for all the systems within 200 seconds upon UV light exposure. The rapid increase of G' compared to G'' is indicative of molecule crosslinking and thus the formation of a solid sample with higher elastic contribution. Despite the difference in G' and G'' , the presence of suspended inorganic phases did not hinder the UV curing of the material. However, as summarised in Table 4.8, even though photocrosslinking was observed for all the systems, COLMA/nano-HA and COLMA/MBG_SG_Sr4% showed slower curing kinetics compared to the system containing only collagen. Comparing the two formulations, after 1200 seconds of UV exposure, COLMA/MBG_SG_Sr4% showed the highest storage modulus and matrix stiffness while the nano-HA containing system proved the lowest crosslinking efficiency. These results suggested that the presence of a second phase can significantly interfere in the process of methacrylated collagen UV curing. This is especially evident in the COLMA/nano-HA, where G' and G'' registered very low values compared to the COLMA system, suggesting once again a more significant interaction between HA and collagen compared to Sr-MBG particles and collagen. On the contrary, MBG_SG_Sr4% nanoparticles seemed to act as reinforcing agents for the collagenous matrix, reaching values of G' up to about 5720 Pa.

Table 4. 8 G' , G'' values and gel time of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems after 100 s and 1200 s of UV light exposure.

	G' , G'' after 100 s (UV 15 mW/cm ²)	G' , G'' after 1200 s (UV 15 mW/cm ²)	Gel time
COLMA	587.6 ± 35.5 Pa 104.6 ± 22.1 Pa	3171.1 ± 199.8 Pa 163.4 ± 18.8 Pa	< 100 s
COLMA/nano-HA	46.9 ± 12.2 Pa 35.3 ± 11.6 Pa	1808.0 ± 122.8 Pa 121.8 ± 25.6 Pa	< 200 s
COLMA/MBG_SG_Sr4%	44.1 ± 9.8 Pa 36.9 ± 12.3 Pa	5720.5 ± 345.8 Pa 98.8 ± 22.4 Pa	< 200 s

Following the study of the UV curing ability and times of COLMA-based systems at room temperature, the suspension printability at 10 °C and the variation of viscosity was analysed to confirm the potential use of the developed systems as biomaterial inks.

In detail, flow ramp tests at 10 °C (the selected printing temperature) were conducted to investigate the variation in the suspension viscosity over a wide range of shear rates (0.01 – 1000 s⁻¹) and to define their yield stress values, as represented in Figure 4.19.

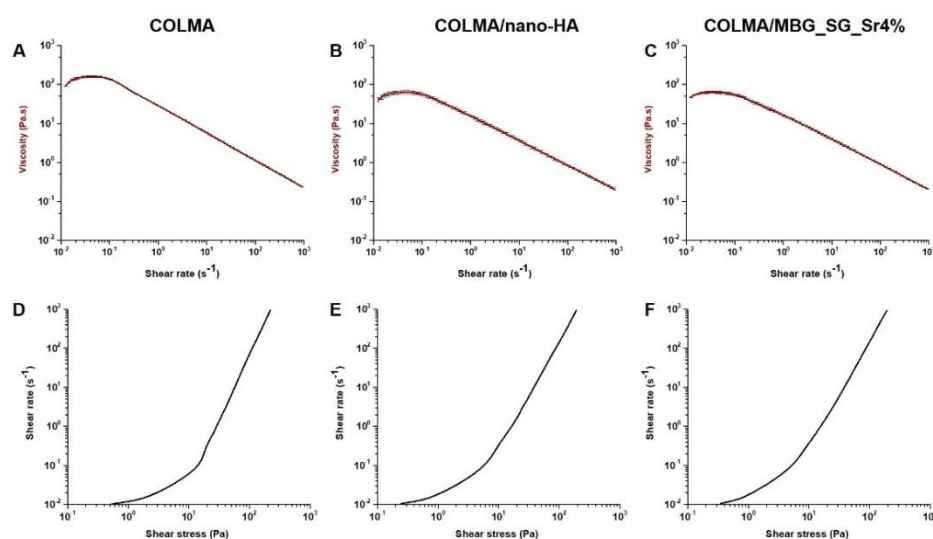


Figure 4. 19 Viscosity and yield stress of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% suspensions at 10 °C.

As visible in Figure 4.19 (A, B, C) all the systems presented a shear thinning behaviour and thus a sharp decrease of the initial viscosity with increasing shear rates, proving the extrudability of the material. As summarised in Table 4.9, the developed COLMA suspensions showed viscosity and yield stress values like those previously reported by the suspensions containing native type I collagen. However, COLMA solution registered the highest value of viscosity compared to the suspensions containing inorganic phases. This aspect may be connected to the collagen chain sliding, promoted by the inorganic particles, whereas the presence of only bulky collagen macromolecules can promote chain entanglement.

Table 4. 9 Viscosity and yield stress values of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% suspensions at 10 °C.

	Viscosity η (max)	Viscosity η (min)	Yield stress
COLMA	160.5 Pa.s	0.3 Pa.s	11.2 Pa
COLMA/nano-HA	63.6 Pa.s	0.2 Pa.s	4.2 Pa
COLMA/MBG_SG_Sr4%	63.2 Pa.s	0.2 Pa.s	3.6 Pa

After performing UV curing tests, the collected solid samples were incubated at 37 °C for 3 hours and then analysed via amplitude sweep tests to investigate the strength of the gel and its potential increase induced by the physical crosslinking of collagen molecules. In details, the value of storage (G') and loss (G'') moduli of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems was measured by means of a dynamic amplitude sweep test (0.01-1% strain) carried out at 37 °C and 1 Hz.

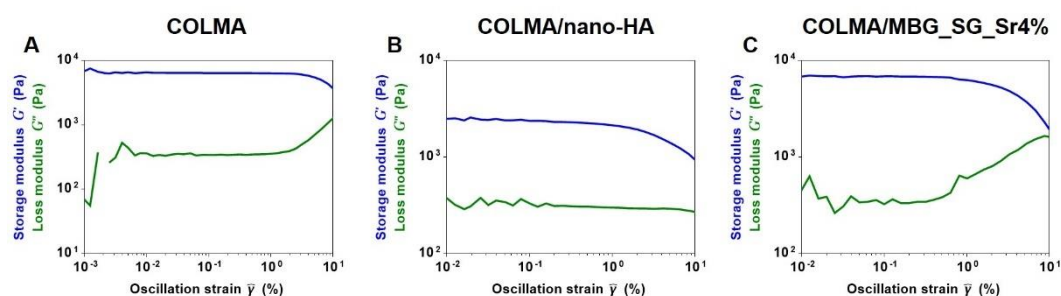


Figure 4. 20 Visco-elastic properties of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems after UV curing and incubation at 37 °C for 3 hours.

Figure 4.20 and Table 4.10 reported the values of G' and G'' for the different analysed systems. All the gels clearly showed a wide linear viscoelastic region between 0.01 and 1% strain, and similar matrix strength, except for the system containing nano-HA. Comparing G' and G'' before and after sample incubation at 37 °C suggested a further crosslinking of the collagen molecules due to the physiological temperature and pH.

The lowest values obtained for the COLMA/nano-HA system were consistent with the results previously obtained for the non-mechacrylated collagen-based system, where the presence of HA partially hindered the collagen assembly mechanisms.

Table 4. 10 G' , G'' values of COLMA, COLMA/nano-HA and COLMA/MBG_SG_Sr4% systems after UV curing and incubation at 37 °C for 3 hours.

	G' after 3h at 37 °C	G'' after 3h at 37 °C
COLMA	6370.1 ± 321.8 Pa	342.6 ± 72.6 Pa
COLMA/nano-HA	2300.0 ± 123.4 Pa	312.2 ± 81.5 Pa
COLMA/MBG_SG_Sr4%	6800.1 ± 388.1 Pa	310.2 ± 88.2 Pa

The resulting data confirmed the potential printability of the systems as well as the effective curing upon application of UV light. However, the time required for the UV crosslinking as well as the viscosity of the suspensions indicated the need to use supporting materials for the 3D printing of the scaffolds. The collected data were thus exploited to optimise the printing process presented in the next section.

4.3.2 3D extrusion printing of UV curable systems

Preliminary tests were performed to assess the printability of the COLMA-based systems by extrusion in the gelatine slurry FRESH V2, following the procedure previously described for the non-methacrylated collagen systems. COLMA/MBG_SG_Sr4% was selected for the preliminary printing assessment based on the enhanced features detected during the rheological characterisation. All preliminary printing tests were conducted with a BIOX 3D Bioprinter equipped with a UV curing printhead, as shown in Figure 4.21.

Before printing, the suspensions were poured in 3 mL UV-shielding cartridges, centrifuged to remove potential embedded air bubbles, and finally inserted in a temperature controlled pneumatic printhead. The suspensions were kept at 10 °C while printing and extruded into the 2 mm thick gelatine slurry FRESH V2 contained in 2-wells chamber-slides, kept at room temperature.

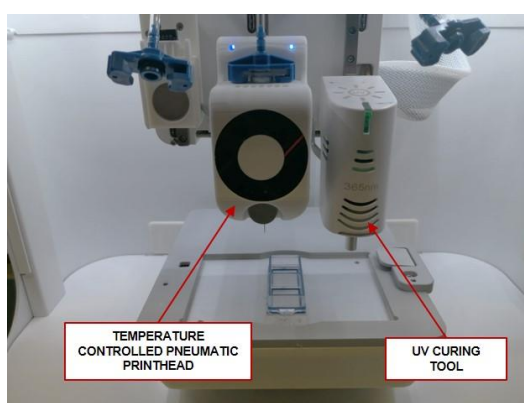


Figure 4. 21 Printing set-up for the preliminary printability assessment of UV curable collagen systems.

To maximise the effect of UV irradiation, the UV curing was set to be active layer by layer, with the UV source centred at the deposited layer. The UV power

was adjusted to 100% (about 15 mW/cm² according to the tool datasheet) selecting an exposure time of 120 seconds and distance from the layer of 1 cm. In summary, the UV curing parameters were selected in order to have the maximum power, the shortest distance and the longest exposure time supported by the printer.

The printing tests were performed with 27 G needles and the parameters optimised by visual observation of the printing resolution of each deposited layer. As shown in Figure 4.22B, honeycomb structures 10 mm x 10 mm, 1 mm thick and with 15%-layer infill were selected to optimise the printing parameters.

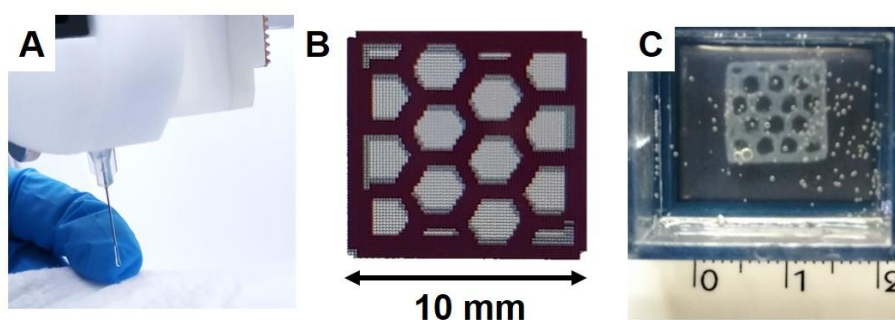


Figure 4. 22 Filament extrusion (A) and printing of honeycomb scaffolds (B, C) using COLMA/MBG_SG_Sr4% suspension.

Starting from the rheological data and the parameters previously optimised for the collagen-based systems, 30 kPa, 11 mm/s and 170 μ m were chosen for pressure, printhead speed and z layer respectively to obtain the best shape fidelity for the Coll/MBG_SG_Sr4% suspension, as represented in Figure 4.22C and reported in Table 4.11. Moreover, the use of COLMA evidenced an overall improvement of the material extrudability compared to the non-methacrylated collagen-based systems, observing the formation of a continuous and homogeneous filament instead of drop-like extrusion upon pressure application (Figure 4.22A).

Table 4. 11. Printing parameters used for the realisation of scaffolds based on COLMA/MBG_SG_Sr4% system.

	Needle	Pressure	Printhead speed	Z layer	Infill (%)
COLMA/MBG_SG_Sr4%	27 G (200 μ m)	30 kPa	11 mm/s	170 μ m	15%

The previous data were presented to confirm the printability of the designed COLMA systems exploiting the optimised FRESH method, described in Section 4.2. However, since the material supporting bath is considered an important obstacle to UV light penetration, specific STL files and G-codes were optimised to enable the simultaneous extrusion of material ink and supporting material, as represented in Figure 4.23.

The designed strategy aims at performing a UV curing step for each layer of deposited material, with a homogeneous exposure and the consequent even reticulation, in order to stabilise the scaffold already after extrusion. The higher

visco-elastic properties demonstrated by the composite after UV light exposure should prevent the scaffold collapse after removal of the gelatine supporting bath.

Based on previous studies conducted by the IRIS research group [29], CAD models reproducing the actual architecture of bone tissue derived by nano-computed tomography analysis on humeral bone samples were obtained and optimised, as visible in Figure 4.23A.

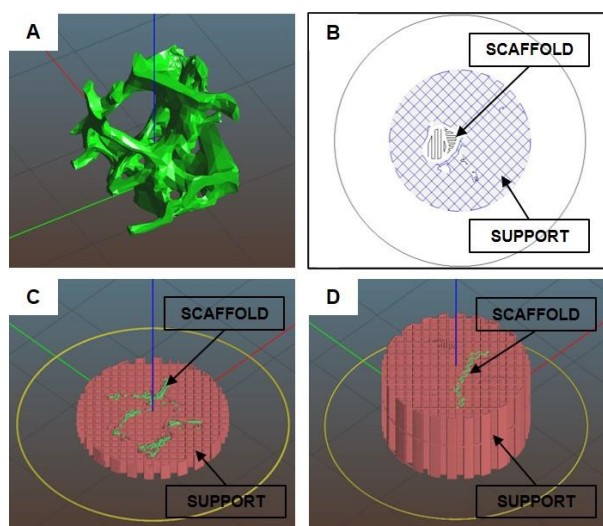


Figure 4. 23 STL file of bone structure (A), 2D section (B) and 3D sections (C, D) of STL files realised by embedding the bone-like structure in the cylindrical model of supporting material.

Subsequently, a Boolean subtraction operation was exploited to create STL files represented by a cylindrical support volume (created according to the dimensions of cylindrical wells in multi-well plates) containing the bone structure rescaled at 4, 6 or 8 mm (Figure 4.23 B, C, D). The resulting file was used to simultaneously print the supporting material and the COLMA/MBG_SG_Sr4% suspension enabling the immediate UV curing of the deposited layer, exploiting the set-up shown in Figure 4.24.

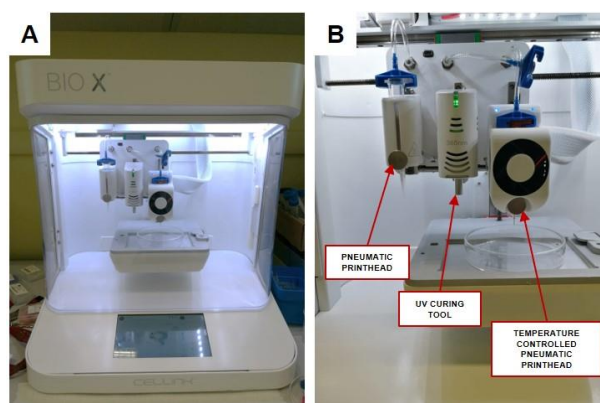


Figure 4. 24 Printing set-up for the design of UV curable scaffolds based on the simultaneous extrusion of scaffold and supporting materials, combined with application of UV light layer by layer.

In details, the extrusion of the gelatine slurry FRESH V2 and the COLMA/MBG_SG_Sr4% suspension was obtained by using a pneumatic

printhead kept at room temperature and a temperature controlled printhead kept at 10 °C, respectively. In parallel, a UV curing tool was exploited to expose the deposited layer to UV light at 365 nm and about 15 mW/cm² for 120 seconds in order to maximise the crosslinking effect. For each layer, the gelatine slurry was firstly deposited in order to provide the proper support for the extrusion of the COLMA suspension and the subsequent UV curing of the material. However, preliminary tests demonstrated a partial dragging of the supporting materials that hindered the accurate deposition of the scaffold segment. According to these observations, the accurate optimisation of the printing parameters for the simultaneous deposition of the different materials is required to avoid potential overlapping between support and scaffolds parts.

In addition, some limitations can arise from technical aspects linked to the 3D printer. According to the manufacturer indications, the UV printhead can expose the layer to UV light up to 120 seconds with the shortest distance of 10 mm, potentially decreasing the curing efficiency. For this reason, the setup of a second UV source for the constant irradiation of the printed structure is currently under evaluation.

4.4 Final considerations

The results presented in this Chapter proved the printability of the developed collagen-based formulations alongside the optimisation of different printing strategies to obtain high-resolution scaffolds.

As reported in the literature, most of the scaffolds based on type I collagen and different inorganic phases for bone tissue engineering are realised by freeze-drying [30–32] or in alternative by the soaking of lyophilised collagen matrices in inorganic phase suspensions [33,34] leading to a limited control over the final shape and porosity. At the same time, hybrid formulations developed for 3D printing applications consider low concentration of inorganic phase in order to promote the proper extrusion of the material, where greater needle diameters are normally preferred to prevent clogging during the process [35,36].

In this work, Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA systems showed their suitability as biomaterial inks for the fabrication of high-resolution 3D printed bone-like scaffolds.

To increase the final printing fidelity while avoiding the printed structure collapse due to the insufficient values of viscosity and yield stress in stationary conditions, the freeform reversible embedding of suspended hydrogels (FRESH method) was exploited and adapted [3]. Based on this technique, the collagen-based materials were extruded in thermo-reversible biocompatible gelatine support bath to enable the deposition of the structure, whereas the subsequent incubation at 37 °C promoted the sol-gel transition of the materials while enabling the removal of the supporting bath.

The printability of the collagenous systems was improved by developing strategies to remove the significant content of air detected, that partially hindered the proper extrusion and deposition of the filaments.

After the proper optimisation of the printing parameters, mesh-like structures with a size of 10 x 10 mm and a thickness up to 5 mm were successfully obtained by using 27 G and 30 G needles, where Coll/MBG_SG_Sr4%/nano-HA demonstrated the best results in terms of shape fidelity.

The use of two different gelatine slurries, FRESH V1 and FRESH V2, was explored comparing the effects on the final morphological features of the printed constructs. Results showed that FRESH V2 favour the printing of more precise constructs, due to its enhanced rheological and physico-chemical properties. Similar observations were obtained also after the chemical crosslinking of the resulting scaffolds by means of GEN/EtOH solutions, where scaffolds realised in FRESH V2 demonstrated to better retain their shape. Moreover, the morphological assessment of the scaffolds of Coll/MBG_SG_Sr4%, Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA by FE-SEM further proved the retention of the homogeneous distribution and organization of inorganic phase particles into the reconstituted fibrillar collagenous matrix.

The FRESH method was thus exploited to obtain high-resolution scaffolds starting from soft biomaterial inks, while the gelatine slurry FRESH V2 was selected as supporting material to perform further experiments. In particular, according to the studies of Lee and co-workers [5], the use of acidic collagen-based suspensions is currently under investigation in order to exploit the buffering capacity of FRESH V2 to induce the fast gelation of the collagen-based material while extruding, finally improving the stability of the printed construct. As a matter of fact, the soft nature of the reconstituted collagen-based scaffolds was considered a significant limitation in the design of more complex architectures, due to the collapse of suspended parts after removal of the supporting material.

An alternative strategy to promote the stabilisation of the extruded material while printing was based on the use of UV curable systems obtained by the combination of methacrylated collagen (COLMA) with MBG_SG_Sr4% and nano-HA.

In the literature, few data are reported about the use of methacrylated collagen as biomaterial ink for the realisation of 3D extrusion printed scaffolds, whereas the main limitations can arise from the proper curing of the material, difficult to achieve during the printing process.

The developed hybrid formulations were comprehensively characterised by means of rheological tests, proving their fast UV curing ability as well as the potential printability due to the shear thinning behaviour. However, the nano-HA containing system demonstrated weaker reconstitution and crosslinking compared to COLMA/MBG_SG_Sr4%. The rheological assessment was also exploited to optimise a final photoinitiator (Irgacure 2959) concentration of 0.5 wt%.

After proving the extrudability of the COLMA/MBG_SG_Sr4% suspension and optimising the process parameters, preliminary printing tests were carried out exploiting the layer-by-layer UV curing of the scaffolds.

STL files were designed to enable the simultaneous extrusion of bone-like structures and supporting materials, enabling the effective application of UV light at each deposited layer. However, the accurate refinement of the printing process

and parameters is still under investigation, in order to promote the proper stabilisation of the material while preserving the precision of the material deposition at each layer. Based on the preliminary results, the optimisation of the process will enable the design of more complex biomimetic structures thanks to the increase of visco-elastic properties during the printing process.

The presented studies thus pave the way for the design of higher complex structures able to reproduce the natural architecture of bone, ideally providing an additional stimulus to direct the regeneration of new healthy tissue.

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CHAPTER 5

Biological assessment of collagen-based formulations and scaffolds: Results and Discussion

5.1 Introduction

This Chapter will report the results derived from the biological assessment of the collagen-based systems developed and optimised for the extrusion printing of bone scaffolds, as described in the previous chapters.

Initially, the cytocompatibility of different chemical crosslinking treatments (4-StarPEG; GEN/PBS; GEN/EtOH) was evaluated on the Coll/MBG_SG_Sr4% system using pre-osteoblast cell lines (MG-63; Saos-2). After the optimisation of Coll/nano-HA and Coll/MBG_SG_Sr4%/nano-HA systems and the selection of the most effective chemical crosslinking method, the developed composite materials and scaffolds were tested in presence of osteoblast (OB) and osteoclast (OC) co-cultures.

The *in vitro* biological assessment of all the designed systems was performed in collaboration with Istituto Ortopedico Rizzoli of Bologna (Scienze e Tecnologie Biomediche, IRCCS) and Università Politecnica delle Marche (Department of Clinical and Molecular Sciences – DISCLIMO) as third parties in the ERC BOOST Project (Biomimetic trick to re-balance Osteoblast-Osteoclast loop in osteoporosis treatment: a Topological and materials driven approach, GA 681798).

In details, cell cultures and experiments using Saos-2 pre-osteoblastic cells were carried out at Università Politecnica delle Marche, while biological tests using MG-63 cells and OB/OC co-cultures were carried out by Istituto Ortopedico Rizzoli.

The cytocompatibility of type I collagen, Sr-MBGs and nano-HA have been widely reported in the literature, alongside their ability to promote bone cell adhesion, proliferation, and differentiation [1–3]. In particular, type I collagen represents a fundamental structural support for different resident cells of soft and hard tissue. Type I collagen can promote cell adhesion thanks to multiple aminoacidic sequences such as GFOGER (glycine, phenylalanine, hydroxyproline, glycine, glutamate, arginine) and RGD (arginine, glycine, aspartate), that work as receptor-recognition motifs able to interact with cells via specific β 1-binding integrins [4,5].

In parallel, bioactive glasses and hydroxyapatite have demonstrated to act as osteoconductive and osteoinductive inorganic phases, potentially increasing the

bioactive character of collagen-based materials, enhancing proliferation and maturation of bone cell precursors both *in vitro* and *in vivo* [1,3,6].

Considering the final application of bone tissue regeneration, chemical crosslinking methods are required to improve mechanical features and stability of the collagen-based matrix and guide the proper cell differentiation [5,7]. Accordingly, scaffolds characterized by higher stiffness, presenting values similar to those reported by the native tissue, have already proved to encourage the cell osteogenic differentiation according to the mechano-transduction pathways [8–10].

However, the toxicity of the common chemical agents, as well as the release of unreacted functional groups, can compromise the cytocompatibility of the resulting constructs, requiring specific assessments [11].

Based on the previous observations, the cytocompatibility of 4-StarPEG crosslinked Coll/MBG_SG_Sr4% was investigated observing cell viability and adhesion of MG-63 pre-osteoblastic cells up to 3 days after seeding. Similarly, the chemical crosslinking performed by means of GEN/EtOH and GEN/PBS solutions was tested on the Coll/MBG_SG_Sr4% system using both MG-63 and Saos-2 pre-osteoblastic cells, comparing cell viability and adhesion between non-crosslinked and crosslinked material samples up to 3 days.

Besides the observations described in Chapter 3 concerning the physico-chemical and mechanical properties of the systems after the different crosslinking treatments, the outcomes derived from these experiments, enabled to select the most effective and biocompatible chemical crosslinking method.

In a second phase and according to the main goals of the ERC BOOST Project, indirect co-cultures of human OBs and OCs were developed by Istituto Ortopedico Rizzoli and subsequently used in combination with the optimised composite systems, to investigate the potential influence over cell proliferation and maturation. The use of cell co-culture represents a key factor in the assessment of new developed biomimetic systems for bone regeneration, enabling the simulation of the natural cell crosstalk mechanisms, reproducing a more effective *in vitro* bone model [12,13].

For the experiments, OBs derived from human trabecular bone were directly seeded on biomaterial and scaffolds, while peripheral blood mononuclear cells (PBMCs – OC precursors) isolated from fresh buffy coat were seeded into transwell systems to set up the indirect co-culture. Cell viability was assessed by means of Alamar Blue assay up to 21 days, in addition to fluorescent and colorimetric assays to define cell maturation and OB adhesion onto the specific substrate.

Firstly, GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA system samples were tested separately to discriminate their influence over OB and OC maturation up to 21 days. Subsequently, the synergistic effect of nano-HA and MBG_SG_Sr4% was preliminary investigated by testing 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA with the OB/OC co-culture system.

5.2 Cytocompatibility of 4-StarPEG crosslinked Coll/MBG_SG_Sr4%

Part of the work described in this section has been previously published [14].

This section will describe the results obtained from the cytocompatibility assessment of the Coll/MBG_SG_Sr4% system before and after the chemical crosslinking with 4-star poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4-StarPEG).

As described in Section 3.5.2, the use of 4-StarPEG as collagen chemical crosslinker was assessed with the aim to increase the stiffness and stability of the Coll/MBG_SG_Sr4% composite material, while preserving the overall cytocompatibility of the resulting system. In this scenario, different studies demonstrated the very low cytotoxic effects of 4-StarPEG, where Moriarty N. and co-workers even proved the high cytocompatibility of cell-encapsulating collagen spheres crosslinked with 4-StarPEG for *in vivo* tissue regeneration [8,15,16].

Despite the low increase in material strength and stability observed after the chemical treatment, as described in Section 3.5.2, the cytocompatibility of Coll/MBG_SG_Sr4% before and after the treatment with 4-StarPEG was explored in order to provide evidence for potential alternative applications.

The biological assessment was performed in presence of MG-63 cell line, observing both viability and adhesion of the cells cultured onto the biomaterial.

The use of immortalized cell lines is more frequent than primary bone cells in the field of applied biology and bone tissue engineering research due to the availability of unlimited amount of cells and more reliable reproducibility by replicate experiments [17,18]. MG-63 cell line has been defined both as the immature osteoblast phenotype model [19] and pre-osteoblastic model [20] and for this reason different authors observed that MG-63 cell type displays a heterogeneous behaviour [21]. This cell model is one of the most used for cytocompatibility studies and it is extremely useful in the first step (pre-screening) of biomaterial evaluation [22].

Initially, the viability of MG-63 cells were investigated at 24h and 72 h after seeding onto biomaterial surface by means of the Alamar Blue assay, commonly used to evaluate cell viability and cytotoxicity of agents [23].

As shown in Figure 5.1, the values obtained by cells seeded onto 4-StarPEG crosslinked Coll/MBG_SG_Sr4% samples were compared to the non-crosslinked system in order to discriminate the biocompatibility of the composite material and the crosslinking treatment.

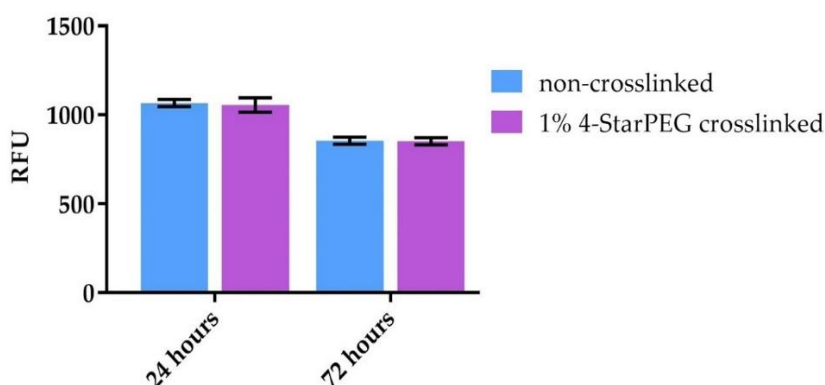


Figure 5. 1 MG-63 cell viability on 4-StarPEG crosslinked and non-crosslinked samples at 24 and 72 h using the Alamar Blue assay.

In detail, the background value of biomaterial without cells seeded, recorded during the reading of the assay (69–94 RFU), was subtracted from the data. Moreover, a control sample represented by the single culture of MG-63 cells without material was considered and analysed, registering a value of about 694 RFU after 24 hours from seeding.

As visible in Figure 5.1, no relevant differences in terms of viability were observed between chemically crosslinked and non-crosslinked samples. Even though no proliferation was observed, cell viability was maintained after 72 h of culture, without registering any significant statistical difference.

Cell viability was thus maintained over time, confirming the overall biocompatibility of the developed composite system. Moreover, the resulting data indicated that the chemical crosslinking reaction by means of 4-StarPEG did not induce the formation and/or the release of toxic components/substances, since MG-63 viability was unaltered after 24 h. Therefore, the absence of cell proliferation, observed at 72 h in both non-crosslinked and crosslinked samples, was not potentially related to the crosslinking procedure or potential residuals.

To better investigate the interaction of MG-63 cells with the substrate, SEM analysis was performed on samples collected after 24 and 72 hours, observing their morphology on both crosslinked and non-crosslinked samples, as reported in Figure 5.2.

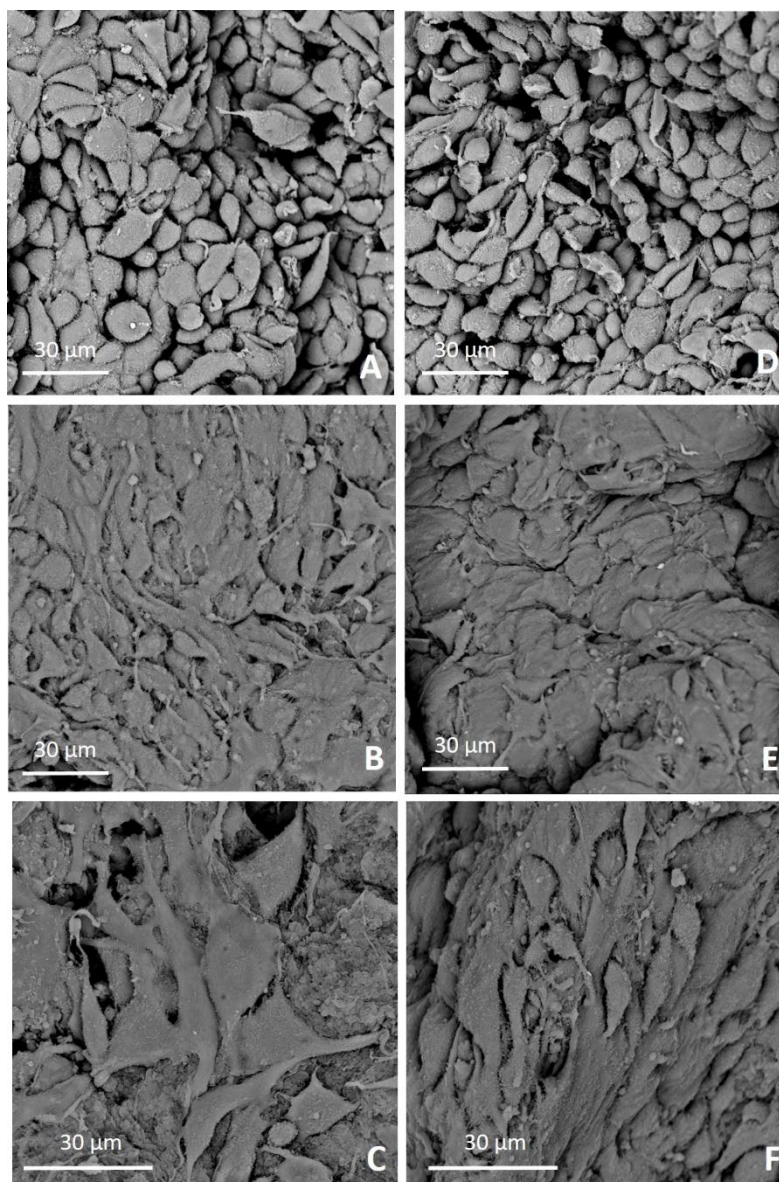


Figure 5. 2 SEM microphotographs of MG-63 adhesion on 4-StarPEG crosslinked Coll/MBG_SG_Sr4% at 24 h (A) and 72 h (B, C), and non-crosslinked Coll/MBG_SG_Sr4% after 24 h (D) and 72 h (E, F).

SEM microphotographs showed a good adhesion of MG-63 on the biomaterial surface at both time endpoints. At 24 h a significant number of cells adhered to the biomaterial surface exhibiting a cuboidal shape (Figure 5.2 A, D), while at 72 h the cells strongly adhered and easily spread showing a flat and elongated shape (Figure 5.2 B, C, E, F).

Prominent filopodia and extracellular protrusions were observed to connect the cells each other and between cells and biomaterial surfaces at 72 h, especially on 4-StarPEG crosslinked Coll/MBG_SG_Sr4% (Figure 5.2 C). Accordingly, cells on the 4-StarPEG crosslinked samples appeared spread and more stretched (Figure 5.2 C) than the ones on the non-crosslinked system (Figure 5.2 F).

These results thus confirmed that even slightly higher stiffness values of the matrix can be detected by cells, favouring a better spreading onto the substrate. Despite the different morphology, MG-63 cells firmly attached to both 4-StarPEG

crosslinked and non-crosslinked samples, denoting a positive interaction between material and cells and thus proving the high cytocompatibility of the system.

5.3 Cytocompatibility of GEN/PBS and GEN/EtOH crosslinked Coll/MBG_SG_Sr4%

Part of the work described in this section has been previously published [24].

As previously described in Section 3.5.3, the use of genipin, a natural chemical agent extract from gardenia fruit (*Gardenia jasminoides* ELLIS), was explored as alternative crosslinking method to obtain higher mechanical stiffness and stability of the developed collagen-based systems. This natural agent is able to induce spontaneous crosslinking of different natural polymers such as collagen, gelatine, and chitosan, and reported a low cytotoxicity and genotoxicity compared to other chemical crosslinking agents [25]. Despite the biocompatibility of genipin was demonstrated to be higher than other common chemical crosslinkers of collagen such as carbodiimides and glutaraldehyde, contrasting results have been presented in different studies, where a common consensus regarding concentration limits and process conditions has not been reached yet [3,6,8,16].

As discussed in Section 3.5.3, the effects of genipin crosslinking were tested using two different solutions: 0.5 wt% genipin in PBS (GEN/PBS) and 0.5 wt% genipin in 70% EtOH, where the latter was used in an attempt to reduce the percentage of strontium ion loss during the crosslinking treatment.

Despite the beneficial effects due to the increased mechanical properties and the reduction of strontium loss, the use of ethanol in cell biology is known to potentially compromise the overall cytocompatibility of the resulting systems.

Therefore, the cytocompatibility of the two different chemical crosslinking treatments with GEN/PBS and GEN/EtOH was assessed comparing the results obtained from non-crosslinked and crosslinked samples of Coll/MBG_SG_Sr4%.

The biological assessment was performed using two types of osteoblast-like cells, MG-63 and Saos-2 cell lines, widely reported as a powerful tool in basic and applied biology field, thanks to the greater cell availability when compared to primary cells, and the consequent reproducibility of results [17,26]. Both are osteosarcoma-derived osteoblasts, but they show different features: MG-63 cells show both mature and immature osteoblastic features, while Saos-2 exhibit a mature osteoblastic phenotype. The use of both cell types allowed an effective investigation of osteoblast behaviour following the preliminary assessment of the cell-material interaction [27].

The cytocompatibility assessment was carried out observing cell viability after 24 and 72 h from cell seeding by means of Alamar Blue assay, while the cell morphology was subsequently analysed through Scanning Electron Microscopy (SEM).

The results of cell viability (Alamar Blue assay) of MG-63 and Saos-2 cultured on GEN/PBS and GEN/EtOH crosslinked Coll/MBG_SG_Sr4% were reported in Figure 5.3.

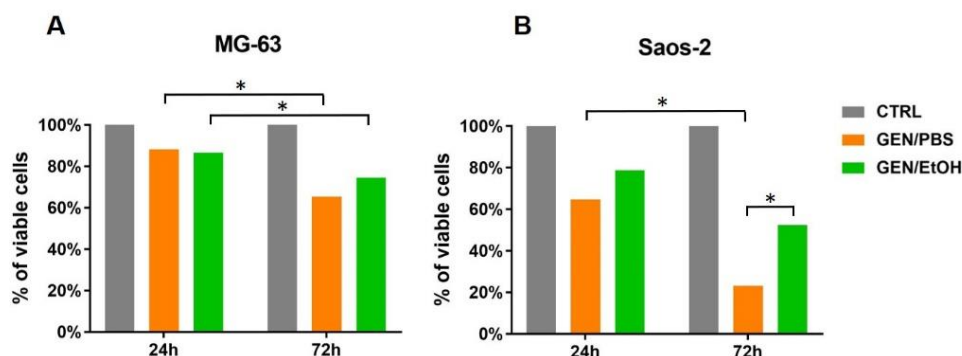


Figure 5. 3 Histograms of Alamar Blue assay showing MG-63 (A) and Saos-2 (B) cell viability on GEN/PBS and GEN/EtOH crosslinked samples at 24 and 72 h (* p-value ≤ 0.05).

After seeding MG-63 and Saos-2 onto material samples prepared as described in Section 2.4.2, the extent of viability was measured subtracting the background value related to the biomaterial without cells (i.e., about 120 RFU) from the RFU of cell-seeded samples. Control of MG-63 seeded onto standard TCPS (CTRL) and analysed at 24 h (907.22 RFU) and 72 h (942.33 RFU) were taken as 100% of viable cells to calculate the percent viability of the other samples.

The reported results confirmed the viability of MG-63 and Saos-2 on both GEN/PBS and GEN/EtOH crosslinked samples after 24 and 72 h of culture, with slightly higher viability found for GEN/EtOH crosslinking in comparison to GEN/PBS. As clearly visible from Figure 5.3, even if cell proliferation was not observed, cell viability was maintained after 72 h from seeding.

At 72 h, Saos-2 reduced their viability on GEN/PBS crosslinked Coll/MBG_SG_Sr4%, whilst they maintained almost similar values on samples treated with GEN/EtOH, although a reduction not statistically significant was observed. A similar trend was observed for MG-63 cells.

Overall, these results indicated that cell viability was maintained over time, confirming the biocompatibility of Coll/MBG_SG_Sr4%, especially when crosslinked with GEN/EtOH.

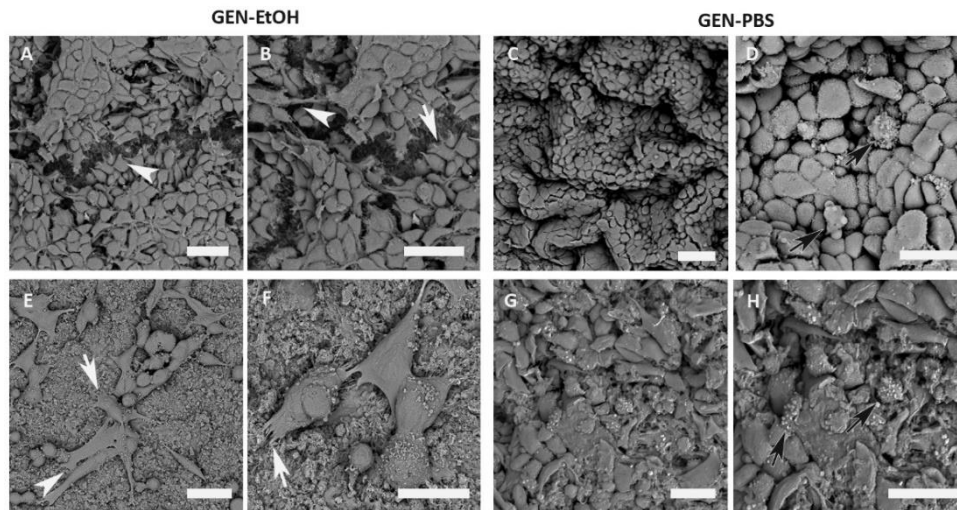


Figure 5. 4 SEM micrographs of MG-63 adhesion at 24 h on 0.5% GEN/EtOH (A, B) and 0.5% GEN/PBS (C, D) crosslinked system; Saos-2 adhesion at 24 h on 0.5% GEN/EtOH (E, F) and 0.5% GEN/PBS (G, H) crosslinked system. Pointed arrows indicate cell–cell contacts, white arrows indicate cell–material interactions and black arrows show suffering features; scale bars 50 μ m.

Subsequently, cell adhesion and morphology of osteoblast-like cells MG-63 and Saos-2 on both GEN/EtOH and GEN/PBS crosslinked systems were assessed after collection at 24 and 72 hours by SEM, as shown in Figure 5.4 and Figure 5.5.

In detail, at 24 h of culture a high number of MG-63 cells adhered to the surface of both GEN/EtOH and GEN/PBS treated samples. On GEN/EtOH crosslinked samples, MG-63 cells appeared flat and more stretched, and contacts among neighbouring cells were evident (Figure 5.4 A, B). On GEN/PBS samples, few points of contact among cells and some round-shaped cells were observed (Figure 5.4 C, D).

Saos-2 cells displayed both round and spread shapes, with several membrane contacts with the material on GEN/ETOH treated samples (Figure 5.4 E, F), while an unusual morphology and evident suffering features with few cell–scaffold connections were observed on GEN/PBS crosslinked system (Figure 5.4 G, H).

As represented in Figure 5.5, after 72 h from seeding, MG-63 cells were still strongly adherent to the GEN/EtOH crosslinked system, showing some cell stretching and branched processes, such as filopodia and membrane protrusions. Similarly, Saos-2 cells displayed fusiform and star-like morphologies and multiple extensions such as lamellipodia and filopodia.

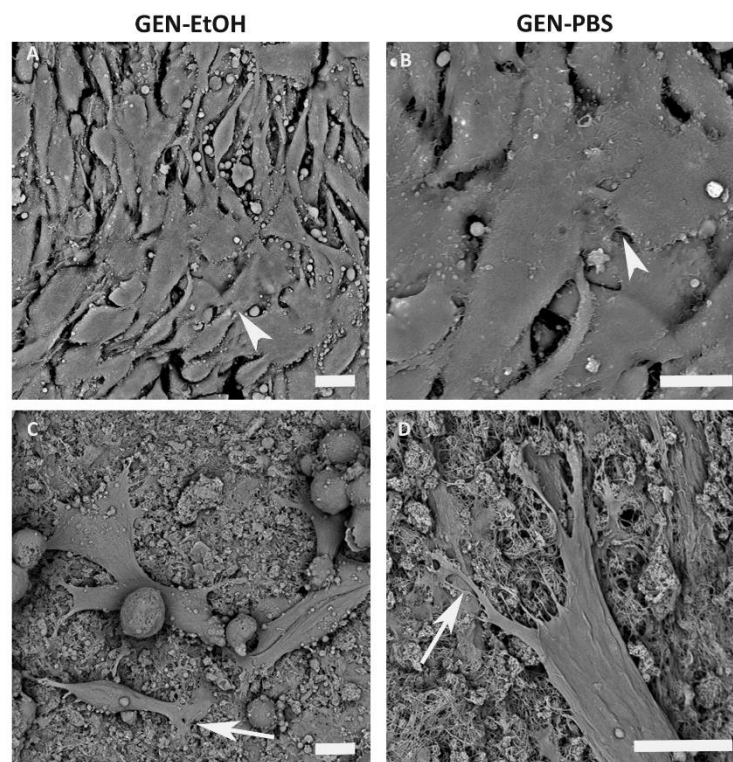


Figure 5. 5 SEM micrographs of MG-63 (A, B) and Saos-2 (C, D) adhesion on 0.5% GEN/EtOH crosslinked system at 72 h. Pointed arrows indicate cell–cell contacts and white arrows indicate cell–material interactions; scale bars 10 μ m.

Considering the results collected for the Saos-2 cells, more adherent cells with a proper morphology and evident cell protrusions were observed on GEN/EtOH in comparison to GEN/PBS.

The reported data confirmed that the crosslinked samples may easily host viable cells, thus evidencing that the chemical crosslinking solutions and the overall process do not lead to any relevant cytotoxic effect.

Morphological analysis highlighted that all samples displayed some surface roughness, an important feature that encourages cellular adhesion, also considered an excellent property for implant integration [28].

MG-63 cells easily attached to the surface of Coll/MBG_SG_Sr4% and remained adherent for both GEN/PBS and GEN/EtOH crosslinked system samples. However, in GEN/PBS treated samples, some MG-63 cells were found to be round-shaped without evident intercellular connections, while on the GEN/EtOH crosslinked system the cells mostly showed a more flattened and stretched shape with some cytoplasmic processes extending to the close cells or the collagen surface.

This different cell response can be due to differences in the mechanical and textural properties of the bioactive material subjected to the two different crosslinking treatments: the higher stiffness presented by GEN/EtOH crosslinked Coll/MBG_SG_Sr4% ($G' = 11032.7 \pm 99.4$ Pa, Section 3.5.3) may, in fact, promote the attachment and spreading of cells, as confirmed in other studies [29].

In parallel, Saos-2 cells cultured on GEN/EtOH crosslinked samples showed superficial irregularities and numerous cytoplasmic processes stretched out to

contact the material, as well as other cells. On Coll/MBG_SG_Sr4% samples crosslinked with GEN/EtOH cells exhibited many filopodia and lamellipodia, whose presence is important in cellular migration and scaffold invasion [30].

For both the osteoblastic-like cell types, the cell adhesion was satisfied over time, and cell–cell and cell–material interactions were observed thanks to the presence of filopodia, lamellipodia and cytoplasmic processes, demonstrating the healthy state of cells and the good interactions with the material surface.

Besides the significant reduction of strontium ion loss during the crosslinking process and the enhanced mechanical stiffness described in Section 3.5.3, a great advantage in the use of GEN/EtOH as chemical crosslinking treatment was therefore demonstrated by the superior biocompatibility confirmed by cell viability and morphology.

Considering the results obtained from the presented biological assessment in addition to the observations described in Section 3.5.3 concerning the physico-chemical and mechanical properties of the material, the chemical treatment with GEN/EtOH solution was selected as the most effective crosslinking method, and further exploited for the crosslinking of the other developed collagen-based composite systems (Coll/nano-HA, Coll/MBG_SG_Sr4%/nano-HA) and scaffolds.

5.4 Osteoblasts and osteoclasts co-culture on GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA

Following the cytocompatibility assessment of the collagen-based systems and crosslinking methods, the influence of the different designed bioactive composites over bone cell adhesion, proliferation and differentiation was assessed and compared. To this aim, this section will describe the preliminary results obtained from the indirect co-culture system of human osteoblasts (OBs) and osteoclasts (OCs) in presence of GEN/EtOH crosslinked samples of Coll/MBG_SG_Sr4% and Coll/nano-HA.

In this context, the use of cell co-cultures instead of single cultures provide an additional value in the screening of new materials developed for tissue regeneration, thanks to the reproduction of a more effective bone-like model.

The coupling of OBs and OCs activities is in fact at the base of the bone remodelling and plays a crucial role in the maintenance of bone volume and skeletal homeostasis, where the correct cellular crosstalk is tightly regulated by systemic factors and molecules locally secreted [12].

Up to now, different OB/OC co-culture models have been proposed to test potential innovative materials thanks to the *in vitro* reproduction of a bone microenvironment, which more closely mimics the natural complex interactions between bone cells [18].

Despite cell lines allow for large-scale expansion and reproducibility, they share only partially the phenotype and properties of the primary cells [31]. Therefore, the use of human primary cells is strongly recommended in the design

of a reliable bone model *in vitro*, even if factors such as variability of the source and poor availability of donor tissue strongly compromise their application [32].

In this frame, different studies aimed at defining the most suitable cell types and culture conditions able to accurately reproduce the bone microenvironment *in vitro*. However, a widely accepted and simple model is still lacking [12]. Accordingly, an indirect co-culture aiming at mimicking the physiological system in which OBs and OCs exchange reciprocal signals, mutually influencing their differentiation, has been developed and optimised at Istituto Ortopedico Rizzoli.

This model, which involves the use of OBs extracted from human trabecular bone and human Peripheral Blood Mononuclear Cells (PBMCs - OC precursors) derived from fresh buffy coat samples, was thus exploited for a preliminary screening of the developed collagen-based systems. This approach allows for the observation of potential interactions between material and native bone cells, stimulating the crosstalk via paracrine communication.

GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples were separately prepared and studied. As schematically represented in Figure 5.6, OBs were seeded onto the surface of the material sample while PBMCs were co-cultured into a transwell device which allows the exchange of paracrine signalling. A basal culture medium (BM) lacking in osteogenic inducers was used for the co-culture experiments in order to better discriminate the potential influence of the material over the cell behaviour and differentiation.

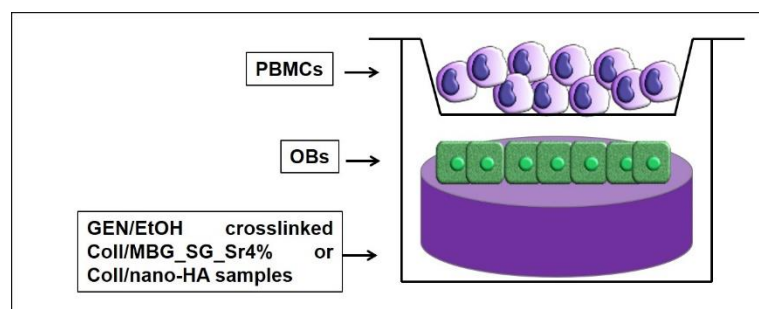


Figure 5. 6 Schematic representation of the co-culture system set-up in presence of GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples.

After cell seeding, different time points (24h, 7d, 14d, 21d) were considered to investigate cell viability as well as proliferation and differentiation up to 21 days, additionally observing OB adhesion onto the biomaterial samples by SEM analysis, as outlined in Figure 5.7.

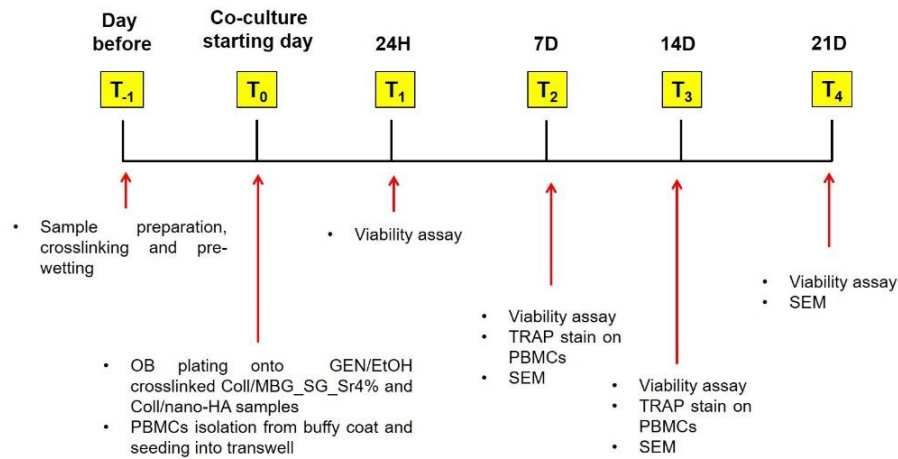


Figure 5. 7 Timeline representing the different steps and timepoints considered during the OB/OC co-culture on GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples.

Both OB and PBMC viability was assessed by means of Alamar Blue assay starting from 24 hours after seeding and up to 21 days, to test the biocompatibility of the bioactive composite materials and the survival of cells in contact with the different samples. As shown in Figure 5.8, the viability of OBs adherent to the biomaterial surface was evaluated separately from the PBMC seeded into the transwell, to detect possible differences in the trend of cell viability.

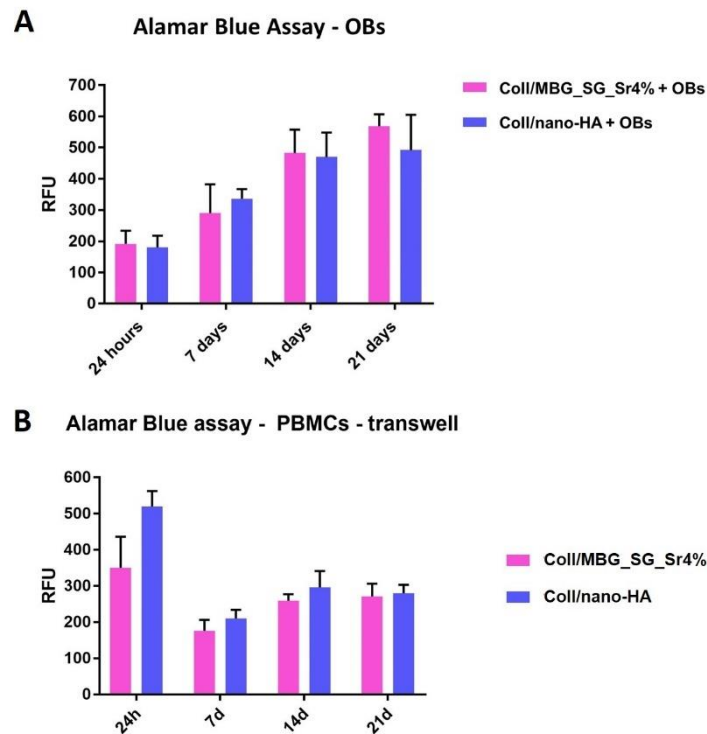


Figure 5. 8 Alamar Blue assay diagram showing viability of OB seeded on biomaterial samples (A) and OC seeded into transwell (B) up to 21 days. Data are expressed as Relative Fluorescence Units (RFU) and reported as mean value + standard deviation.

The reported results successfully demonstrated high values of cell viability over time for both GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH

crosslinked Coll/nano-HA, without observing significant differences in RFU values at each timepoints.

The viability of OBs seeded onto both biomaterial samples presented an increasing trend up to 21 days, suggesting high proliferation rates, where slightly higher values were obtained in the case of GEN/EtOH crosslinked Coll/MBG_SG_Sr4% samples.

On the contrary, the number of viable PBMCs showed a constant decrease over time where a significant drop was registered after 7 days from seeding in transwell. Regarding the two different composite systems, GEN/EtOH crosslinked Coll/nano-HA overall demonstrated to induce higher viability of PBMC up to 14 days after seeding. The evident decrease in their viability from day 7, suggested the potential detachment of cells seeded into transwell systems, due to the frequent medium change. Moreover, the potential formation of multinucleated mature OCs from a higher number of mononucleated cells over time, could also explain the measurement of an overall lower signal. In addition, the higher number of viable OB cells as well as the lower viability of PBMC in presence of MBG_SG_Sr4%, seemed to suggest their ability to favour OB proliferation and maturation and the simultaneous inhibitory effect over OC vitality, as reported in some studies present in the literature [33–35].

In summary, the Alamar Blue assay indicated high biocompatibility of the two tested collagen-based systems, that were also able to support a significant proliferation of osteoblasts adherent to the biomaterial surface.

As further evidence of cell vitality, Live-dead assay was performed on OB seeded on both GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples up to 21 days.

After staining, the collected samples were visualised under fluorescent microscope to detect the extent of viable cells represented by a green colour and dead cells depicted as red.

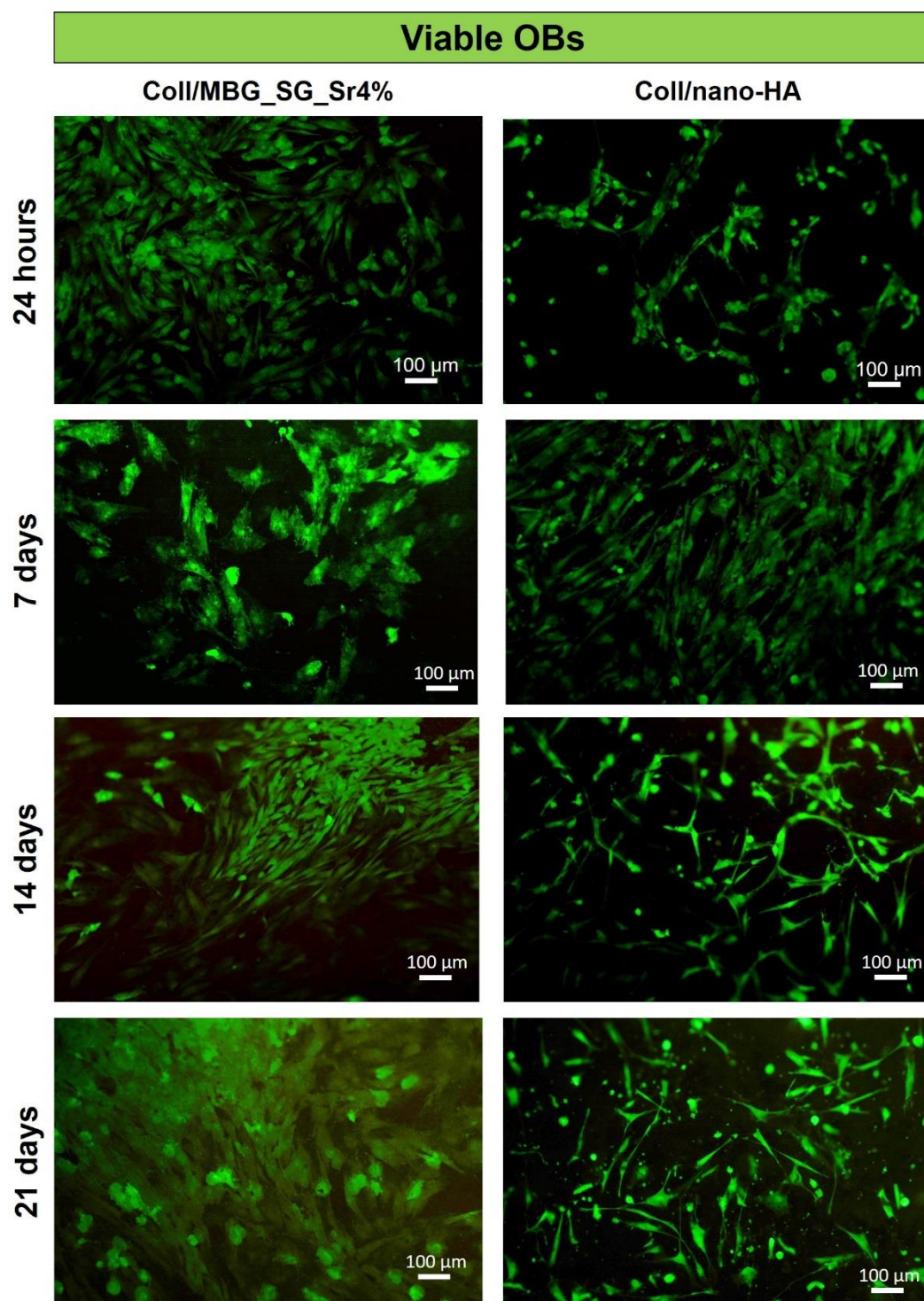


Figure 5. 9 Representative images of viable OBs stained with Live Dead assay and seeded on GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA at the different time points considered (24h, 7d, 14d, 21d).

Images collected from the two composite systems at each time point (24h, 7d, 14d, 21d) were analysed and compared to detect potential differences of cell viability and morphology, as illustrated in Figure 5.9.

The results of Live/Dead confirmed the previous obtained by Alamar Blue assay, demonstrating good cell adhesion and high viability already after 24 hours from seeding.

Since the number of dead cells was irrelevant, the reported images represented only viable cells, to enable a better definition of cell morphology.

The high viability of cells was thus confirmed for both GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA up to 21 days. Moreover, OBs seeded onto Coll/MBG_SG_Sr4% samples were observed to be larger and more flattened compared to OBs seeded onto Coll/nano-HA, that presented a spindle-shaped morphology. A larger cuboidal shape normally suggests a greater cell maturation, whereas the elongated morphology presented by OBs seeded onto Coll/nano-HA samples may be favoured by the rod-like shape characteristic of nano-HA particles.

The morphology and adhesion of OB seeded onto the two different composite systems was further investigated by means of SEM analysis, as represented in Figure 5.10.

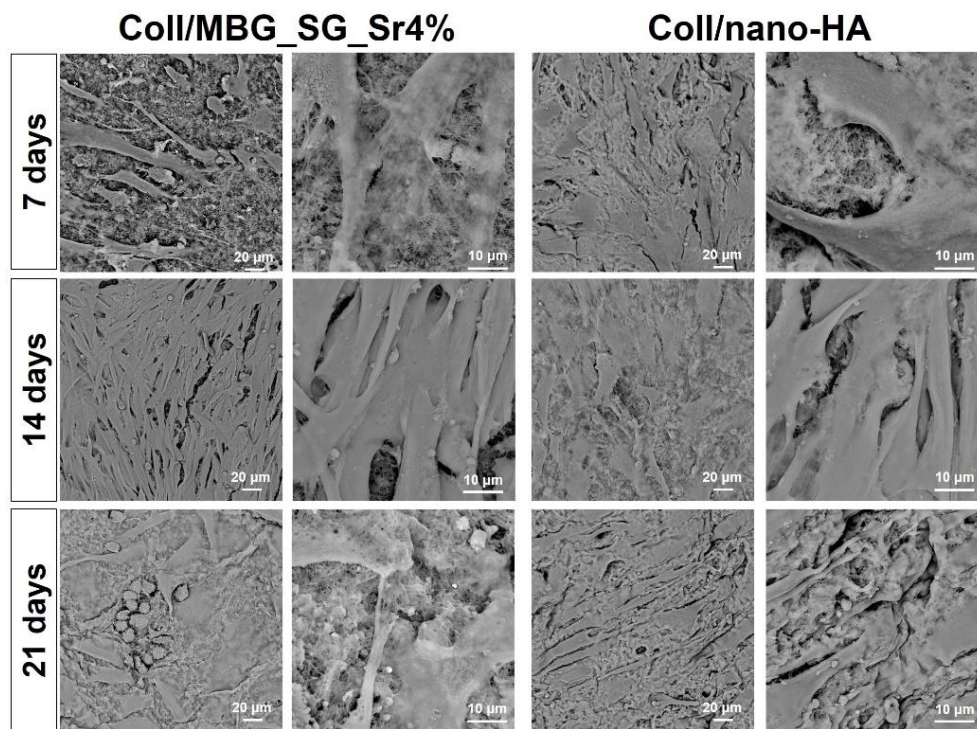


Figure 5. 10 SEM images of OB adhesion onto GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples after 7, 14 and 21 days.

SEM images further demonstrated the successful cell adhesion on both Coll/MBG_SG_Sr4% and Coll/nano-HA samples, as well as the evident cell proliferation at increasing time points. Evident interactions between cells as well as material and cells were detected, with no significant differences between MBG_SG_Sr4% or nano-HA containing systems. On the contrary, a slight change in cell morphology was observed after 21 days of seeding comparing OB seeded onto Coll/MBG_SG_Sr4% and Coll/nano-HA surfaces. According to the images collected from Live/Dead assay, OBs seeded onto Coll/MBG_SG_Sr4% showed a larger and more polygonal shape, suggesting a more mature phenotype, potentially favoured by the presence of MBG_SG_Sr4% particles. This result seemed to be

supported also by the detection of regular globular elements measuring about 20-30 μm in size after 21 days of seeding. According to different studies in the literature, round-shaped agglomerates can represent calcified matrix particles resulting from calcium binding osteocalcin secreted by OBs [36,37]. On the contrary, the globular accretions were not visible on samples of Coll/nano-HA, where cells resulted more elongated, according to the previous observations.

Besides OB vitality and morphology, the differentiation of PBMC/OC-precursors in presence of the two different composite systems was assessed by means of different colorimetric assays.

To this aim, Phalloidin-fluorescein isothiocyanate (FITC) and Hoechst 33258 stains were exploited to track cell actin filaments and nuclei, respectively.

Considering the time normally required for OC maturation, PBMCs seeded onto transwell were collected and stained at 7 and 14 days of co-culture and analysed under fluorescent microscope, as represented in Figure 5.11.

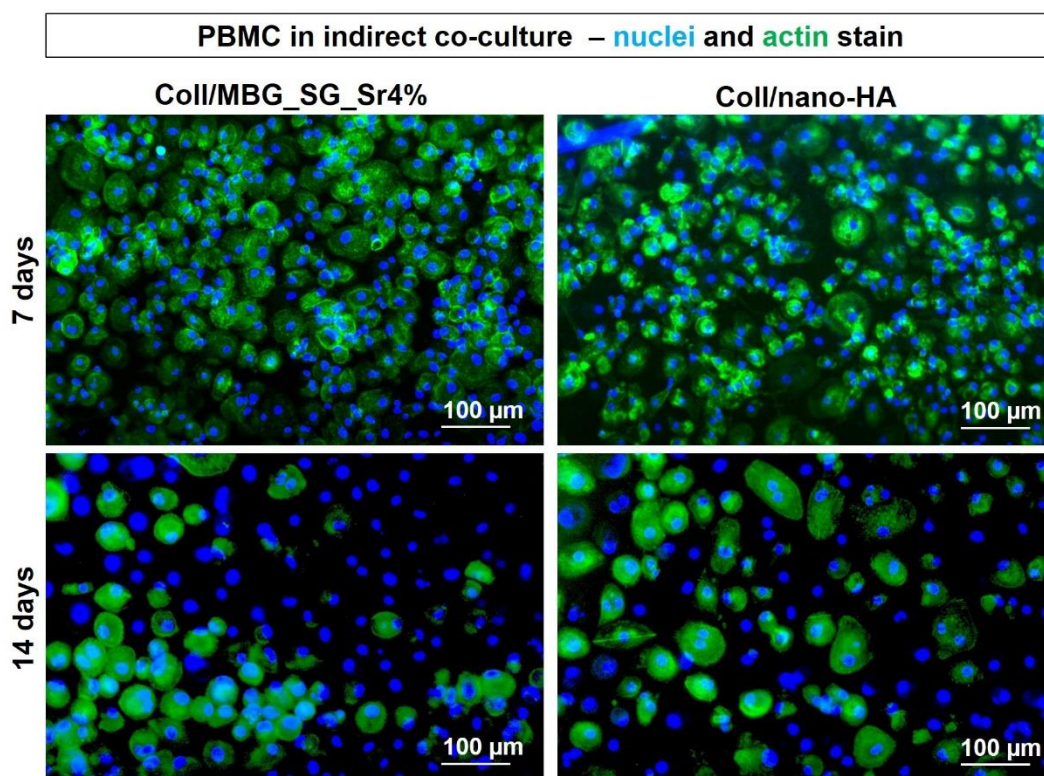


Figure 5. 11 Phalloidin-FITC and Hoechst 33258 stain to highlight actin filaments and nuclei in PBMCs seeded into transwell and in indirect contact with OB seeded GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples after 7 and 14 days of coculture.

The differentiation of PBMCs toward OC-precursors in indirect co-culture with OB-loaded materials was thus assessed observing the development of actin filaments and the potential formation of multinucleated cells.

Despite PBMCs were seeded onto PET membranes (transwell) and cultured in basal medium (BM) without the presence of any osteogenic inducing factor, some multinucleated cells with positive stain for actin were detected for both co-culture with GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA systems. No significant differences were observed for the two

different composites, where images collected after 14 days of co-culture were overall indicative of higher cell maturation.

The results obtained were consistent with those reported by experiments performed on 2D OB/OC co-cultures and carried out by Istituto Ortopedico Rizzoli, where the maturation of PBMC and the formation of multinucleated cells in basal medium conditions was observed after 14 days from seeding, whereas faster and more evident differentiation was detected when using osteogenic medium. However, the differentiation capability is not comparable to other culture conditions due to the singularity of the different co-culture systems reported in the literature [12].

To further investigate the differentiation of PBMC towards OC-precursors over time, samples collected at 7 and 14 days were stained for tartrate-resistant acid phosphatase (TRAP), as represented in Figure 5.12.

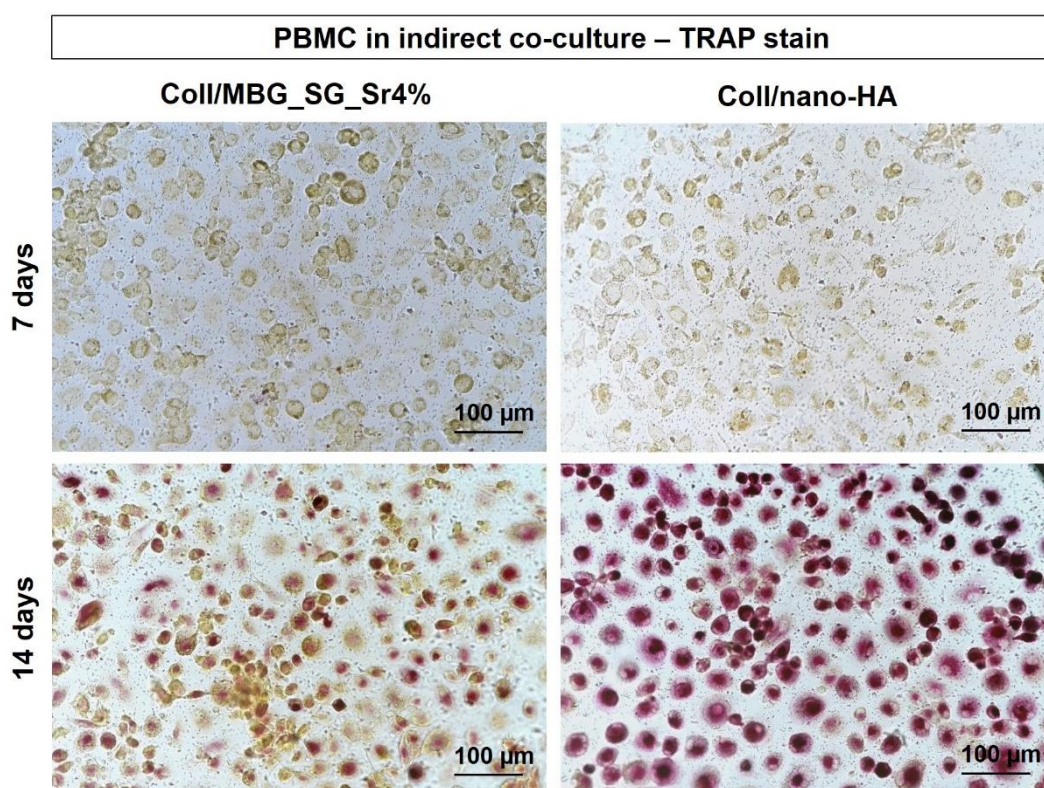


Figure 5. 12 TRAP stain to highlight the production of the enzyme in PBMCs seeded into transwell and in indirect contact with on OB seeded GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and GEN/EtOH crosslinked Coll/nano-HA samples after 7 and 14 days of coculture.

TRAP-stained spots positive for the enzyme production were detected in both PBMC samples co-cultured with OB seeded on Coll/MBG_SG_Sr4% and Coll/nano-HA systems after 14 days, according to the greater cell maturation suggested by fluorescent staining. Moreover, higher positive signals were detected for cells cultured in indirect contact with GEN/EtOH crosslinked Coll/nano-HA, where the presence of nano-HA particles seemed to promote a faster differentiation of OC-precursors cells, supporting the previous observation about the partial inhibitory effect induced by MBG_SG_Sr4%.

The preliminary results obtained from the biological assessment with OB/OC co-cultures, proved the high biocompatibility of both Coll/MBG_SG_Sr4% and Coll/nano-HA composite systems, and their ability to support OB adhesion and proliferation as well as OC-precursors differentiation, without evidencing significant differences between the two 3D co-culture systems. Despite the promising results obtained, further investigation is required with a particular focus on the detection of cell maturation markers and gene expression, with the aim to additionally discriminate the potential different influence induced by the bioactive inorganic phases.

Based on the preliminary outcomes and considering the similar physico-chemical features of the two composite systems, the presence of MBG_SG_Sr4% suggested to higher stimulate the proliferation and maturation of OBs, while the nano-HA containing formulation seemed to promote a faster differentiation of PBMCs towards mature OCs.

5.5 Osteoblasts and osteoclasts co-culture on 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA

Following the results obtained by the biological assessment of the two composite systems i.e., GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA, this section will describe the preliminary evaluation conducted on the indirect co-culture of OBs and OCs in presence of 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA.

In this phase, the potential contribution of both MBG_SG_Sr4% and nano-HA to cell viability, proliferation and differentiation was tested using the developed Coll/MBG_SG_Sr4%/nano-HA system. Moreover, the use of a more organised 3D environment was explored, where OBs were seeded directly onto 3D printed scaffolds (10 x 10 x 1 mm) characterised by pores of about 1 mm, as represented in Figure 5.13.

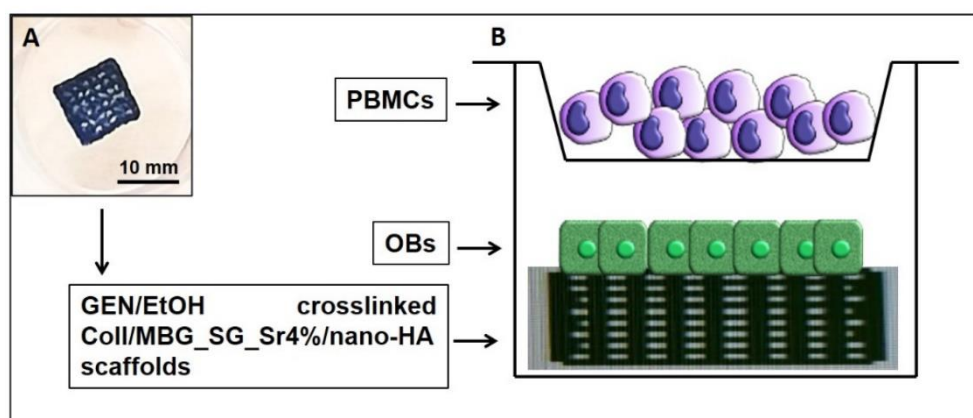


Figure 5. 13 3D printed scaffold of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA (A) and schematic illustration of the OB/OC coculture system (B).

3D scaffolds were produced by extrusion printing of the Coll/MBG_SG_Sr4%/nano-HA suspension into the gelatine slurry FRESH V1 and further chemically crosslinked with GEN/EtOH solution, as described in section 2.2.12.

As described in Figure 5.14, PBMC derived from fresh buffy coat samples were placed into transwell to set up the indirect co-culture, while OBs were seeded onto the scaffold surface after performing a prewetting treatment. Similarly to the previous experiments, basal medium lacking in osteogenic factors was used for the co-culture system, with the aim to better discriminate the potential influence of the designed scaffold over cell proliferation and differentiation.

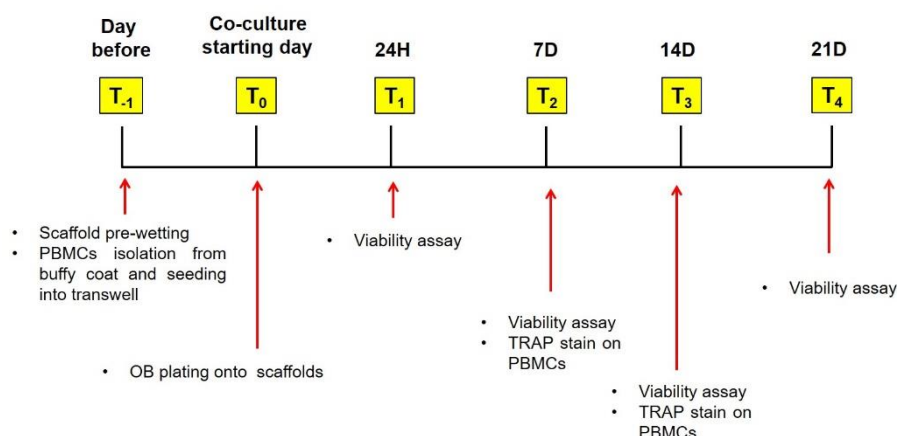


Figure 5. 14 Timeline presenting the different steps and timepoints considered during the OB/OC coculture on 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA.

The viability of both OBs and OCs was assessed at different time point (24h, 7d, 14d, 21d) by means of Alamar Blue and Live/Dead assay up to 21 days, while the extent of PBMC maturation was explored by means of fluorescent and TRAP staining.

As shown in Figure 5.15, the viability of OBs adherent to the scaffold and PBMCs seeded into the transwell, were evaluated separately in order to better define the main differences.

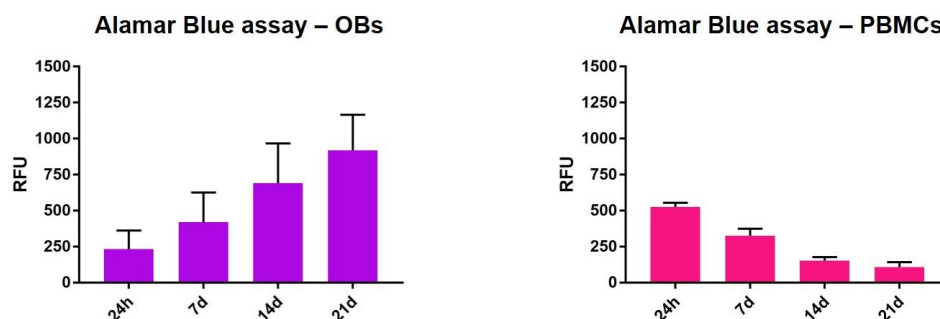


Figure 5. 15 Alamar Blue assay diagram showing viability of OB seeded on scaffolds (left) and OC seeded into traswell (right) up to 21 days. Data are expressed as Relative Fluorescence Units (RFU) and reported as mean value + standard deviation.

The results from Alamar Blue assay demonstrated a significant increase in cell viability over time for OBs seeded onto the scaffold surface, while an opposite trend

was observed for PBMCs. The biocompatibility of the composite system as well as the manufacturing process was therefore confirmed by the high number of viable cells detected up to 21 days, where the constant increase of viable OBs suggested a marked rate of proliferation.

As previously discussed for the separate systems of Coll/MBG_SG_Sr4% and Coll/nano-HA, the decrease in cell viability observed for PBMCs in indirect co-culture can be partially ascribed to the culture conditions. In particular, both the polyethylene terephthalate (PET) transwell membrane used for the seeding and the multiple steps of medium refresh can compromise both adhesion and vitality of PBMC over time.

To confirm the data derived from Alamar Blue, Live/Dead assay was performed on OB seeded onto the scaffold surface up to 21 days. Moreover, as visible in Figure 5.16, the staining with calcein-AM (green reagent for viable cells) was exploited to observe the morphology of OB adherent to the substrate.

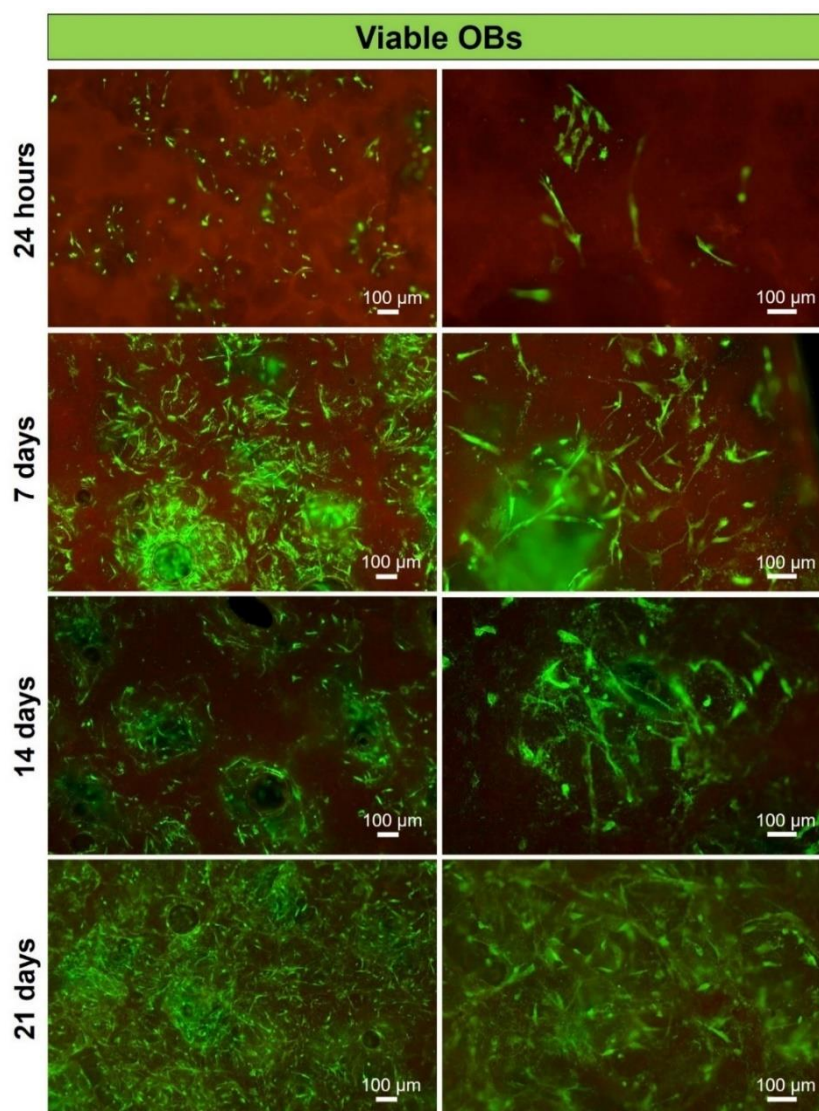


Figure 5. 16 Representative images of viable OBs stained with Live Dead assay and seeded on 3D printed scaffolds GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA at the different time points considered (24h, 7d, 14d, 21d).

At the different timepoints, the stained samples were observed under optical fluorescence microscope, revealing the extent of viable and dead cells. As evident from the images reported in Figure 5.16, the number of dead cells was irrelevant compared to viable cells, confirming the proliferative trend registered by the Alamar Blue assay up to 21 days.

In terms of morphological features, the increasing number of OBs attached onto the scaffold surface also showed a more elongated and spindle shape, as previously observed for OBs cultured onto the nano-HA containing system.

Osteoblast adhesion is recognised to be highly influenced not only by the biochemistry, but also by the morphological features of the substrate. The collected results therefore demonstrated that the designed scaffold favoured the proper adhesion and proliferation of cells, fundamental for the subsequent triggering of signalling pathways regulating both OB and OC activities [13].

Besides the assessment of OB viability and adhesion onto 3D printed scaffolds of Coll/MBG_SG_Sr4%/nano-HA, the potential differentiation of PBMC was evaluated after 7 and 14 days of co-culture.

In detail, Phalloidin-fluorescein isothiocyanate (FITC) and Hoechst 33258 fluorescent stains were exploited to observe actine filaments and nuclei of OC-precursor cells, as shown in Figure 5.17.

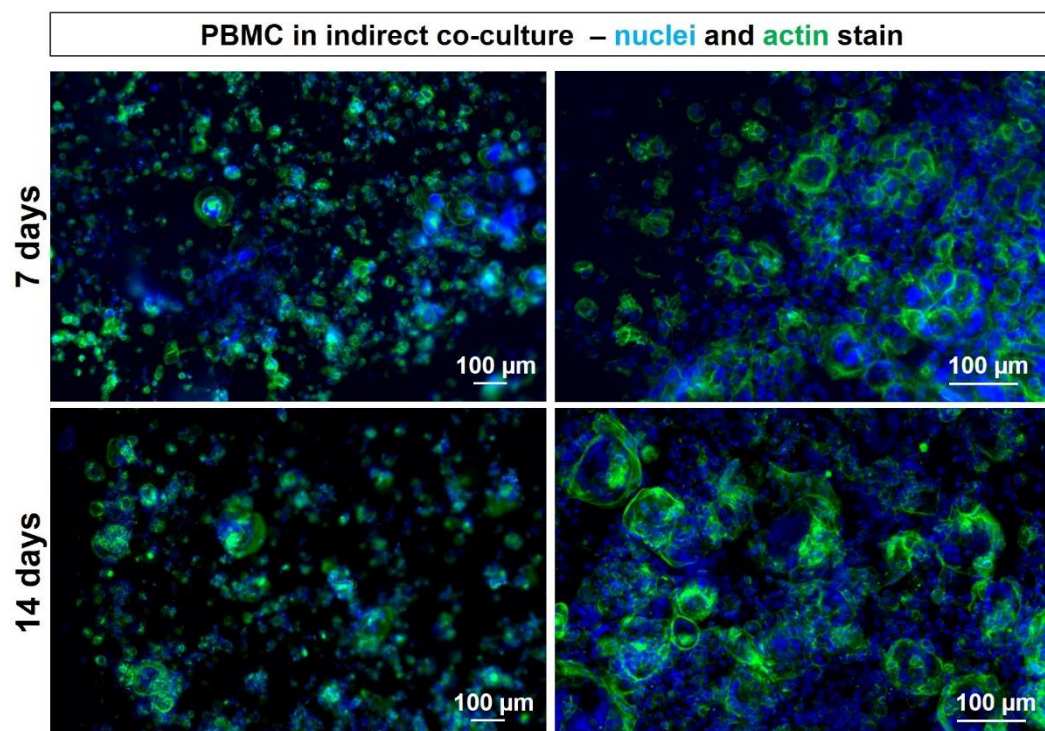


Figure 5. 17 Phalloidin-FITC and Hoechst 33258 stain to highlight actin filaments and nuclei in PBMCs seeded into transwell and in indirect contact with OB seeded onto 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA after 7 and 14 days of coculture.

Particularly after 14 days of co-culture, images proved the presence of multinucleated cells presenting a larger actin ring, suggesting the positive development towards a more mature phenotype, despite the absence of osteogenic inducing factors. The use of basal medium further supported the potential positive

effect induced by the composite scaffold over the final differentiation of PBMCs towards mature OC-precursors.

Finally, to confirm cell maturation, PBMC were stained for TRAP after 7 and 14 days from seeding, as represented in Figure 5.18.

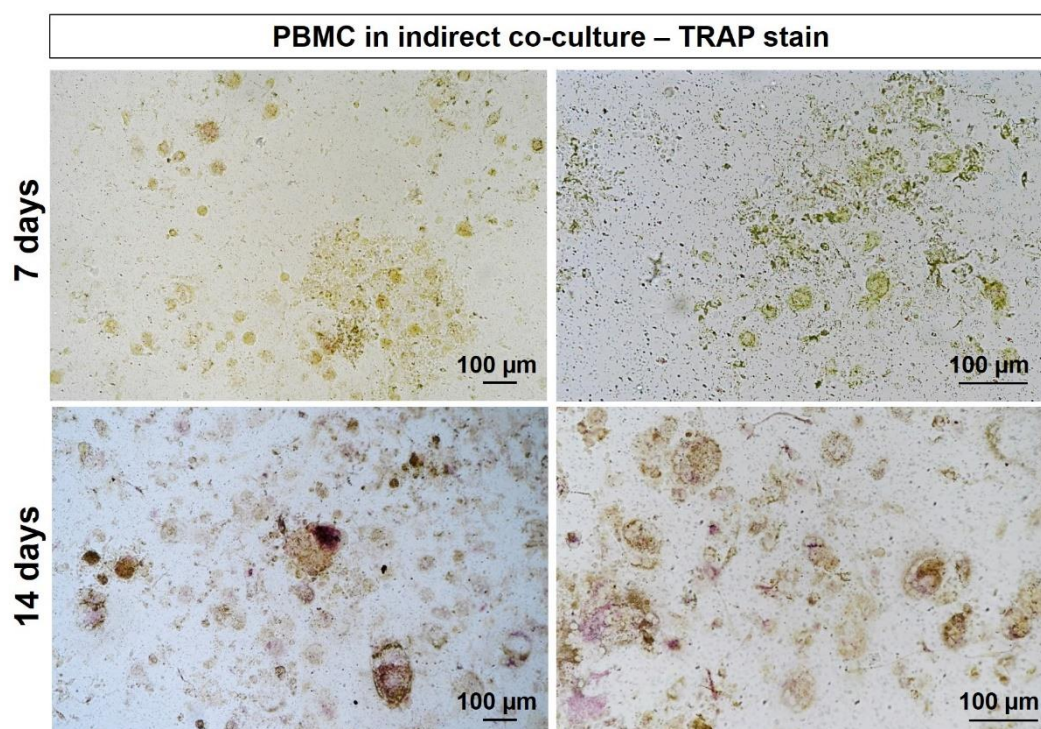


Figure 5. 18 TRAP stain to highlight the production of the enzyme in PBMCs seeded into transwell and in indirect contact with OB seeded onto 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA after 7 and 14 days of coculture.

TRAP-stained samples revealed the presence of dark spots related to the production of the enzyme only after 14 days of seeding, demonstrating a higher maturation of cells. In particular, the reported images resulted to be more similar to those obtained at 14 days for PBMC co-cultured in presence of Coll/MBG_SG_Sr4% system, where a lower positive signal for TRAP compared to the nano-HA containing system was detected. These data seemed to suggest that a higher amount of nano-HA in the composite may promote a faster differentiation of PBMC for osteoclast function.

The preliminary results obtained from the indirect OB/OC co-culture applied to the 3D scaffolds therefore enabled a first screening regarding the biocompatibility of the developed system, also confirming the overall cytocompatibility of the process optimised to obtain the 3D printed constructs.

Despite the positive outcomes in terms of cell viability and proliferation, as well as the detection of clear signals of cell differentiation in absence of additional osteogenic inducers, further investigation is required to better understand the influence of the developed system over cell response, particularly discriminating the stimulus offered by MBG_SG_Sr4% and nano-HA bioactive phases.

5.6 Final considerations

The results obtained by the biological assessment and presented in this Chapter, confirmed the biocompatibility of all the collagen-based bioactive composites developed, and were exploited to define the most effective chemical treatment for the stabilisation of materials and scaffolds. Moreover, indirect OB/OC co-cultures were exploited to perform a preliminary screening of the optimised crosslinked collagen-based systems and scaffolds, demonstrating their ability to support cell proliferation and maturation.

In the first phase of the study, pre-osteoblastic MG-63 and Saos-2 cell lines were used to explore the cytocompatibility of the first optimised composite system, i.e., Coll/MBG_SG_Sr4%, and the different chemical crosslinking treatments. In this frame, MG-63 and Saos-2 cell lines were used instead of primary cells due to the greater cell availability and the higher reproducibility of results, according to different studies in the literature [17,26].

The tests, performed in collaboration with Istituto Ortopedico Rizzoli of Bologna (Dr. G. Ciapetti, Prof. N. Baldini) and Università Politecnica delle Marche (Prof. M. Mattioli-Belmonte), highlighted the overall biocompatibility of both material and crosslinking treatments explored, demonstrating cell viability and adhesion up to 72 hours. Moreover, the collected data evidenced a greater viability of cells seeded onto GEN/EtOH crosslinked Coll/MBG_SG_Sr4% substrates, where cells showed a more spread and elongated morphology as well as higher cell-material interactions. According to these results and considering the enhanced mechanical stability induced by the chemical treatment, the GEN/EtOH solution was selected as most effective crosslinking agent and was further used to increase the properties of the other developed collagen-based constructs.

Based on the demonstrated cytocompatibility of both material and chemical crosslinking method, GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA systems were tested for their ability to support viability, proliferation and maturation of human OB and OC cells. To this aim, an indirect OB/OC co-culture system optimised by Istituto Ortopedico Rizzoli using OBs extracted from human trabecular bone and human Peripheral Blood Mononuclear Cells (PBMCs - OC precursors) derived from fresh buffy coat samples, was exploited for the pre-screening of the different bioactive composites.

As discussed in Chapter 3, the bioactive collagen-based systems were developed in an effort to mimic both chemical and morphological features of native bone ECM, further exploiting additive manufacturing technologies to impart a structural stimulus at different length scales. In this scenario, the use of human cell co-culture systems represents a key tool in regenerative models, able to reproduce a more reliable bone microenvironment that can be exploited for the pre-screening of new materials and scaffolds, to better understand the different stimuli provided by the engineered constructs [12].

Accordingly, the co-culture model was set up by the simultaneous seeding of OB cells onto the different biomaterial samples, while PBMC were placed into transwell, in indirect contact with the OB/material system. In addition, basal culture medium lacking in osteogenic inducers was used to better discriminate the potential influence of the material over cell behaviour and differentiation.

Cell viability and proliferation was monitored up to 21 days from seeding, while after 7 and 14 days the potential maturation of OC-precursors was assessed by means of different colorimetric assays. Both GEN/EtOH crosslinked Coll/MBG_SG_Sr4% and Coll/nano-HA proved to support OB viability and proliferation up to 21 days, as well as strong adhesion and spreading on both substrates. These results agree with different studies in the literature reporting that, bioactive and hydrophilic surfaces presenting nano-scale roughness represent ideal substrates for OB cells adhesion, promoting the formation of focal points that determine the reorganisation of their cytoskeleton, where cells decrease their height and expand the surface of contact. The adhesion mechanisms are therefore considered key factors, since they regulate cell survival, migration, and differentiation [13].

In contrast to what observed for OB cells, PBMC exhibited a decreasing trend of viability up to 21 days after seeding, especially for the MBG_SG_Sr4% system, where the release of strontium could additionally act as negative factor for cell viability, as well as the seeding onto the transwell membrane.

Despite the lower values of viability, PBMC suggested to develop a more mature phenotype after 14 days of culture due to the observation of some multinucleated cells as well as the production of TRAP enzyme, more evident in presence of Coll/nano-HA.

The experiments overall evidenced slight differences between the MBG_SG_Sr4% and nano-HA containing composites. In detail, a slightly higher OB viability as well as a more cuboidal and larger morphology were observed in presence of MBG_SG_Sr4% particles, while nano-HA containing systems evidenced a more marked maturation of OC-precursors after 14 days of co-culture. These outcomes suggested a potential positive influence of strontium ion release from MBG particles, as well as their ionic dissolution products (Si, Ca), over OB survival, proliferation, and maturation, whereas a partial inhibitory effect on PBMC activity was observed, compared to the nano-HA containing system. The ability of strontium ions to promote osteoblast replication while downregulating OC activities, has been already reported in different studies in the literature [33,34,38]. Further investigation is required to provide more reliable evidence with the aim to better discriminate the singular contribution of MBG_SG_Sr4% and nano-HA.

In parallel, the same OB/OC co-culture model was tested in presence of 3D printed scaffolds of GEN/EtOH crosslinked Coll/MBG_SG_Sr4%/nano-HA, as pre-screening of the designed constructs. In this case, the synergistic effect of both bioactive inorganic phases as well as the ability of cells to adhere to the scaffold structure were explored up to 21 days.

A high viability and proliferation rate over time was measured for OB seeded onto the scaffold surface, confirming the biocompatibility of the system alongside the overall manufacturing process. However, as previously observed, a decrease trend of viable PBMC was measured at increasing time points.

As for the results obtained from the separate composite systems, Coll/MBG_SG_Sr4%/nano-HA scaffolds proved to support cell viability and proliferation, as well as the differentiation of PBMC towards a mature phenotype.

These preliminary outcomes indicated the suitability of the developed OB/OC co-culture model for the investigation of the regenerative potential of bioactive materials and constructs, also demonstrating the high biocompatibility of the designed systems. Different aspects focused on differentiation signals and genic expression are currently under investigation to achieve a more in-depth understanding of the different stimuli provided by the designed biomimetic systems.

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CHAPTER 6

Collagen-based scaffolds fabricated by means of electrospinning: Results and Discussion

6.1 Introduction

This Chapter will present the development of collagen-based formulations for the design of biomimetic scaffolds by means of electrospinning techniques for bone tissue engineering applications.

The research activities have been conducted in collaboration with the Institute for Technology-Inspired Regenerative Medicine (MERLN) in the frame of the GIOTTO European Project (Active aGeIng and Osteoporosis: The next challenge for smarT nanobiOmaterials and 3D technologies; NMBP-22-2018, GA 814410).

Besides 3D printing technologies, electrospinning represents a promising tool to produce biomimetic scaffolds since it enables the production of nanofibrous constructs mimicking the features of the extracellular matrix at the micro- and nanoscale [1,2]. The process consists in the deposition of dry fibres starting from polymeric solutions that are extruded upon application of an electric field, where the deposition and the morphological features of the resulting mats can be partially controlled by the optimisation of the process parameters as well as the physico-chemical properties of the materials [1,3,4].

Despite the chance of creating biomimetic nanostructured scaffolds, the use of collagen for electrospinning applications widely reported critical aspects, due to the potential denaturation of the protein caused by the harsh solvents and process parameters employed [1,5,6].

In this frame, the use of different non-toxic solvents as well as high collagen concentrations has been explored in order to obtain homogeneous fibrous scaffolds, whilst maintaining the integrity and physico-chemical properties of native collagen [2,7]. Different electrospinning process conditions have been subsequently investigated in order to better discriminate the contribution of each parameter to the final realisation of the fibrous spun matrix, always exploiting a plate collector and an emitter of 21 G (0.8 mm internal diameter). Morphological assessment by means of Scanning Electron Microscopy (SEM) was used to investigate the morphology of all the spun matrices and measure the fibre diameters, defining the influence of the different process parameters as well as the physico-chemical properties of the material over the final features of the scaffold. The resulting data led to the

definition of the best collagen formulation and process conditions to obtain homogeneous nano-fibrous mats.

With the aim to obtain a homogeneous hybrid formulation suitable for electrospinning applications, the introduction of MBG_SG_Sr4% nanoparticles in the previous optimised collagen system was explored. Preliminary tests were conducted to confirm the processability of the suspension with electrospinning technologies and the good dispersion of the inorganic phase into the fibrous matrix, to form biomimetic composite mats with great potential for bone tissue engineering applications.

6.2 Optimisation of material formulation and process parameters for the design of collagen-based electrospun scaffolds

This section will be focused on the optimisation of the material formulation and process parameters to design collagen nano-fibrous scaffolds by means of electrospinning technology.

The choice of solvent and polymer concentration plays a critical role in the scaffold fabrication alongside electrospinning parameters such as flow rate, voltage, and collector distance. Solvents normally used for electrospinning applications have been found to significantly influence the native physico-chemical properties of collagen, while the process conditions mainly affect the final scaffold morphology and characteristics [3].

The electrospinning of natural polymers have reported different complications due to their high viscosity and low solubility, often requiring the addition of synthetic polymers or the use of organic toxic reagents [2].

Despite several studies reported the successful electrospinning of collagen when combined with highly volatile fluoroalcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) or 2,2,2-trifluoroethanol (TFE), the use of these solvents proved to induce an evident denaturation of the protein native structure [1,2]. At the same time, non-toxic solvents such as ethanol or PBS solutions often led to the formation of mats with larger and less homogeneous fibres, often presenting high concentrations of deposited salts in the final scaffolds [8].

Considering the effects of these solvents on the native ultrastructure of collagen, more environmentally benign and less harsh systems were explored to obtain collagen electrospun scaffolds [5]. Accordingly, acidic-based solvents were evaluated to allow collagen solubility also at high concentrations as well as the native structure preservation.

As starting point and based on the previous studies conducted by Ribeiro and co-workers [2], a concentration of 120 mg/mL of collagen was dissolved in a solution of acetic acid (AA), ethyl acetate (EA) and distilled water (dH₂O) with a

ratio of 4:3:3 (AA/EA/dH₂O) at 4 °C. The obtained system will be reported hereafter as 12%COL in AA/EA/dH₂O.

The addition of ethyl acetate was explored with the aim to increase the conductivity of the solution, favouring the formation of a stable electrostatic jet and Taylor cone. In addition, limiting the water component has been previously demonstrated to be beneficial to improve the spinnability and avoid high surface tension that usually contributes to the poor processability of water-based polymeric solutions [2,3,5].

Different stirring times as well as different electrospinning process conditions were investigated in order to better discriminate the contribution of each parameter to the final realisation of the fibrous spun matrix.

To produce the scaffolds, the collagen solution was processed for 20 minutes using a home-made electrospinning setup composed of a high voltage power supply and a syringe pump regulating the material flow, connected to the emitter of 21 G (0.8 mm internal diameter). A grounded plate collector was used for the experiments and was covered with aluminium foil to promote the easy removal of the resulting mats.

Different process conditions such as applied voltage, material flow, distance between emitter and collector plate and environmental conditions (temperature and relative humidity recorded in the electrospinning chamber) were varied to obtain a stable flow and the deposition of homogeneous nanofibers of collagen.

The scaffolds were then collected and analysed by means of Environmental Scanning Electron Microscopy (ESEM) in order to define their morphological features according to the parameters set, as shown in Figure 6.1.

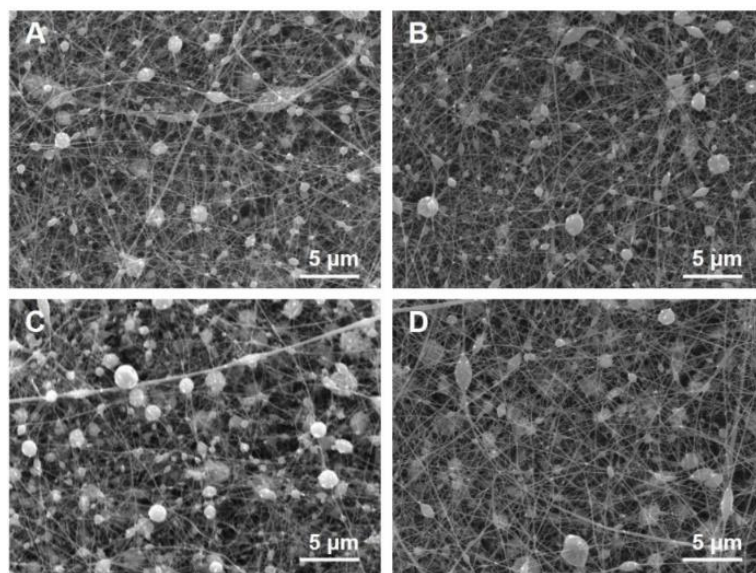


Figure 6. 1 ESEM images of 12% COL in AA/EA/H₂O scaffolds obtained using different process parameters (see Table 6.1).

As expected, the electrospinning process yielded a randomly orientated and interconnected fibrous mesh at the nano-scale. As shown in Figure 6.1, the 12% COL in AA/EA/dH₂O system led to the formation of non-homogeneous fibrous

matrices and the presence of beads of small and big size up to 2 μm , according to the poor jet stability observed during the process.

No significant differences were detected comparing the different process parameters tested and all the scaffolds were characterised by fibres below 250 nm in diameter. Moreover, longer stirring times were observed to poorly influence the solubility and the final processability of the collagen solution.

Table 6. 1 Electrospinning parameters used for 12% COL in AA/EA/H₂O system (see Figure 6.1).

	Voltage	Flow	Collector distance	Environmental conditions	Material processing	Collagen concentration
A	20 kV	0.1 mL/h	15 cm	23°C; 40% humidity	OVERNIGHT STIRRING	12 wt%
B	20 kV	0.05 mL/h	15 cm	23°C; 40% humidity	OVERNIGHT STIRRING	12 wt%
C	20 kV	0.1 mL/h	15 cm	23°C; 40% humidity	48H STIRRING	12 wt%
D	20 kV	0.05 mL/h	12 cm	23°C; 40% humidity	48H STIRRING	12 wt%

According to previous observations regarding the successful increase of the solution stability for greater polymer concentrations [3,9], the collagen content in the final system was thus increased to 200 mg/mL. The collagen powders were then dissolved in AA/EA/dH₂O (4:3:3) overnight at 4 °C and the final solution was subsequently electrospun at different process conditions as reported in Table 6.2.

The obtain system will be reported as 20% COL in AA/EA/dH₂O.

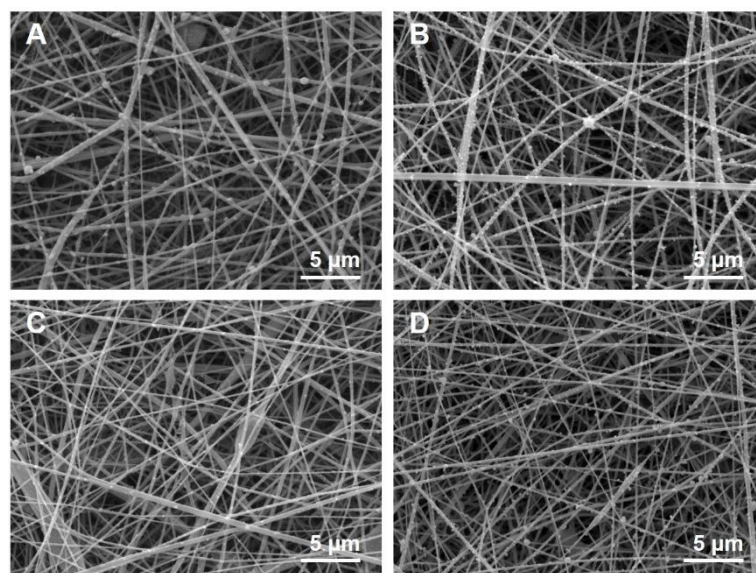


Figure 6. 2 ESEM images of 20% COL in AA/EA/H₂O scaffolds obtained with different process parameters (see Table 6.2).

ESEM images of the different spun matrices showed the effective formation of more homogeneous fibres with a diameter ranging between 100 and 400 nm for all the conditions tested. However, as shown in Figure 6.2 the formation of beads up

to 0.5 μm was still observed in all the samples except for the solution processed at 20 kV, 0.05 mL/h and 12 cm of collector distance (Figure 6.2C). However, these conditions formed matrices containing a significant amount of ribbon-like fibres, overall decreasing the homogeneity of the scaffold.

Table 6. 2 Electrospinning parameters used for 20% COL in AA/EA/H₂O system (see Figure 6.2).

	Voltage	Flow	Collector distance	Environmental conditions	Material processing	Collagen concentration
A	20 kV	0.1 mL/h	15 cm	23°C; 40% humidity	OVERNIGHT STIRRING	20 wt%
B	20 kV	0.05 mL/h	15 cm	23°C; 40% humidity	OVERNIGHT STIRRING	20 wt%
C	20 kV	0.05 mL/h	12 cm	23°C; 40% humidity	OVERNIGHT STIRRING	20 wt%
D	20 kV	0.1 mL/h	12 cm	23°C; 40% humidity	OVERNIGHT STIRRING	20 wt%

Despite the improved results in terms of fibre morphology and homogeneity, both 12% and 20% COL in AA/EA/dH₂O (4:3:3) systems showed evident phase separation (Figure 6.3) and instability after about 1 hour at 23 °C, highlighting potential issues for longer times of processing.

Despite similar material formulations and process parameters were reported to form homogeneous nanostructures, the contrasting results obtained in this study may be ascribed to the different type I collagen origin and purity, as well as environmental conditions such as relative humidity of air and temperature, as previously observed [4].

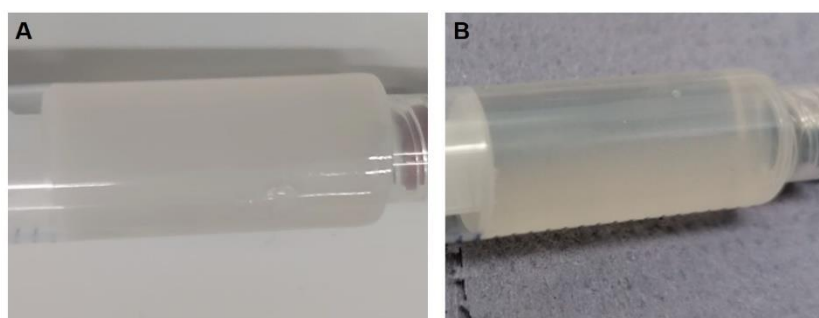


Figure 6. 3 Image showing the 20% COL in AA/EA/H₂O solution immediately after preparation (A) and the phase separation after 1 hour of processing (B).

The inhomogeneous mats obtained using AA/EA/dH₂O (4:3:3), as well as the instability of the polymeric system thus suggested the necessity of finding alternative solvents to create more stable solutions.

In this frame, different studies in the literature proved that high concentrations of acetic acid in water enhanced the overall solubility of collagen molecules alongside its spinnability, forming homogeneous fibres below 100 nm in diameter using concentration ranging between 25 wt% and 30 wt% [4].

Therefore, based on the procedure reported by Ting Liu et al. [7], a concentration of 250 mg/mL of collagen was dissolved in 40% acetic acid solution (40% AA) overnight at 4 °C, obtaining a final system named hereafter as 25% COL in 40% AA.

Rheological analyses on collagen solutions in AA/EA/H₂O (3:2:2) and 40% AA were performed to compare the viscosity and the overall processability of the systems, as reported in Figure 6.4.

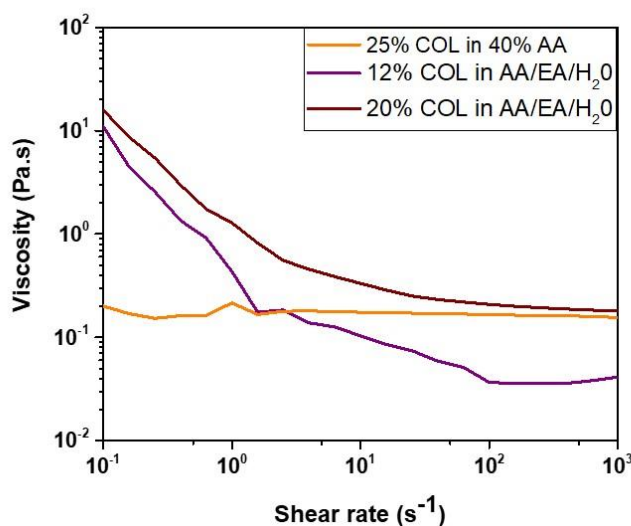


Figure 6. 4 Comparison between flow ramps obtained from 12% COL in AA/EA/H₂O, 20% COL in AA/EA/H₂O and 25% COL in 40% AA at 23 °C.

As shown in the graph, 12% and 20% COL in AA/EA/H₂O systems showed a shear thinning behaviour and higher values of viscosity at almost zero-shear conditions. On the contrary, a higher concentration of collagen (25 wt%) dissolved in 40% AA showed a Newtonian behaviour, characterised by constant and lower values of viscosity in the wide range of applied shear rates.

These results confirmed the key role of acetic acid in promoting the dissolution of collagen molecules, associated with lower viscosity values, as previously reported [5]. Accordingly, the higher values of viscosity registered for collagen dissolved AA/EA/H₂O suggested a lower solubilisation of the protein.

25% COL in 40% AA was thus processed by electrospinning, varying high voltage, flow rate, collector distance and environmental conditions until observing a constant and stable material jet, as summarised in Table 6.3.

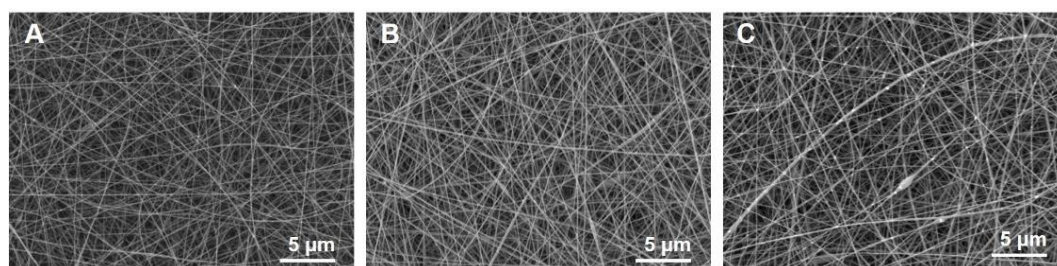


Figure 6. 5 ESEM images of 25% COL in 40% spun scaffolds obtained for different process parameters (see Table 6.3).

The morphological analysis by means of ESEM, shown in Figure 6.5, revealed the formation of homogeneous nanometric fibres in the range of 100 – 300 nm for all the process conditions with a limited detection of beads or swollen fibres.

In particular, the solution spun at 20 kV, 0.05 mL/h and 15 cm of collector distance showed the best results, giving a resulting fibrous matrix with single mode fibres of about 100 nm.

Table 6. 3 Electrospinning parameters used for 25% COL in 40%AA system (see Figure 6.5).

	Voltage	Flow	Collector distance	Environmental conditions	Material processing	Collagen concentration
A	20 kV	0.05 mL/h	15 cm	23°C; 40% humidity	OVERNIGHT STIRRING	25 wt%
B	20 kV	0.1 mL/h	12 cm	23°C; 40% humidity	OVERNIGHT STIRRING	25 wt%
C	20 kV	0.05 mL/h	12 cm	23°C; 40% humidity	OVERNIGHT STIRRING	25 wt%

The high solubility of collagen molecules in acid solutions and organic solvents is due to the absence of covalent bonds between triple helices of collagen after extraction from native tissues [4]. Higher solubility of collagen is normally preferred to produce more stable and homogeneous solutions, thus allowing the electrospinning and the achievement of homogeneous sub-micrometric fibres.

However, as previously reported, the use of harsh solvents and strong acids has been suggested to hinder the natural folding of collagen into triple helix, almost absent in the resulting electrospun scaffolds [1–4].

In this frame, the influence of AA/EA/H₂O and 40% AA solvents over the final supramolecular structure of collagen was investigated by means of Circular Dichroism, observing the potential decrease in the triple helical fraction.

The influence of the solvents alongside the electrospinning process was defined comparing native collagen powders with collagen reconstituted after dissolution in both AA/EA/H₂O and 40% AA, and electrospun scaffolds obtained by the processing of 25% COL in 40% AA, as shown in Figure 6.6.

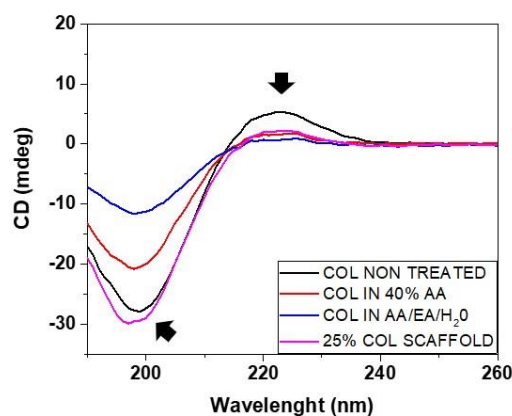


Figure 6. 6 Circular dichroism analysis performed on native collagen (COL NON-TREATED), collagen reconstituted after dissolution in AA/EA/H₂O (COL in AA/EA/H₂O), collagen reconstituted after dissolution in 40% AA (COL in 40%AA) and collagen scaffolds obtained by processing 25% COL in 40% AA (25% COL SCAFFOLD).

The spectrum derived from original collagen powders (black curve) displayed a sinusoidal pattern with a deep negative peak at about 198 nm, a cross-over around 215 nm and an evident positive peak at 220 nm, indicating the presence of a triple helical configuration [8]. In details, the negative peak is characteristic of randomly arranged α -chains while the positive one corresponds to the triple helix structure, whose intensity decreases upon denaturation [1,4].

In parallel, collagen reconstituted after dissolution in 40% AA (COL in 40%AA) and electrospun scaffolds obtained by processing 25% COL in 40% AA (25% COL SCAFFOLD) exhibited similar trends, comparable with that reported by native collagen, showing an evident negative peak at about 198 nm and a weakened positive band at 222 nm.

On the contrary, collagen reconstituted after dissolution in AA/EA/H₂O solvent (COL in AA/EA/H₂O) lacked the positive peak, as normally reported for gelatine and denatured collagen, due to the disruption of the triple helix conformation and the progressively dissociation into the randomly coiled α -chains.

These results evidenced that the use of 40% AA and the further processing with electrospinning led to a greater preservation of the ultramolecular structure of collagen compared to systems obtained from AA/EA/H₂O and other solvents reported in the literature. These observations are in accordance with previous studies in the literature that reported higher preservation of the supramolecular structure of collagen when using acetic acid solutions [1,4].

Based on the previous results, 25% COL in 40% AA was selected for further investigation of the developed collagenous system in combination with inorganic phases in order to obtain biomimetic composite scaffolds for bone tissue engineering.

6.3 Collagen-based composite scaffolds by means of electrospinning: preliminary assessment

Based on the positive results obtained, the 25% COL in 40% AA system was selected to further incorporate inorganic particles to obtain biomimetic composite scaffolds.

Considering the few data reported in the literature regarding the combination of collagen and mesoporous bioactive glasses for the design of scaffolds by means of electrospinning, the use of MBG_SG_Sr4% was explored with the aim to obtain bioactive and biomimetic collagen-based composites. The introduction of inorganic phases in the final system is crucial to obtain biomimetic structures, mimicking the composite nature of bone tissue while reproducing its architecture at the micro- and nano-scale, exploiting electrospinning techniques.

Above all, the most straightforward strategy to include nanoparticles into the electrospinning process is to disperse them into the polymer solution. In order to allow a uniform distribution and avoid particles aggregation, nanoparticles were sonicated for 6 min using a horn sonicator in water and then dispersed in the polymer solution.

As preliminary assessment and based on previous studies reporting the combination of polymeric solutions with different inorganic phases [3,6,9], MBG_SG_Sr4% were mixed with collagen to reach a final concentration of 5 wt%, obtaining a homogeneous 25% COL/MBG_SG_Sr4% in 40% AA system.

Before electrospinning, the viscosity of the resulting suspension was registered and compared with that previously registered for 25% COL in 40% AA, as reported in Figure 6.7.

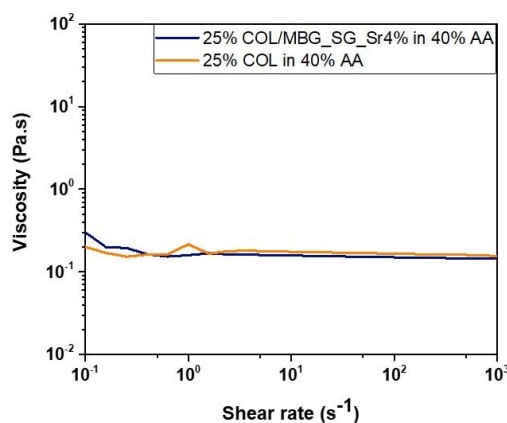


Figure 6. 7 Viscosity of 25% COL in 40% AA and 25% COL/MBG_SG_Sr4% in 40% AA at 23 °C.

Figure 6.7 showed the viscosity of collagen solution and its suspension subjected to a wide range of shear rate applied. As clearly visible, the collagen-based suspension showed a Newtonian behaviour, registering low and constant values of viscosity, as observed for the 25% COL in 40% AA solution. Therefore, the introduction of MBG_SG_Sr4% did not alter the rheological properties of the

system, suggesting a good dispersion of the nanoparticles as well as weak interactions between the inorganic and the organic phases [6].

Considering the similar rheological properties registered for the collagen solution and the hybrid formulation (Figure 6.7), the electrospun composite scaffolds were obtained by using the same process parameters previously optimised and defined for the 25% COL in 40% AA system.

The morphological features of the resulting scaffolds and the dispersion of the inorganic phase was explored by means of ESEM analysis, as shown in Figure 6.8.

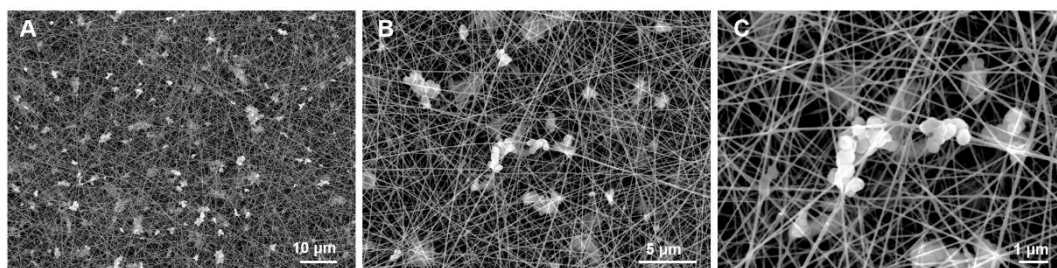


Figure 6. 8 ESEM images of 25% COL/MBG_SG_Sr4% scaffolds at different magnifications.

The most homogeneous scaffolds were fabricated using 20kV voltage, flow rate of 0.1 μ L/h at a distance of 12 cm from the collector.

The resulting mats showed fibres ranging from 100 nm to 130 nm, which is comparable to the fibre size formed during the electrospinning of 25% COL mats. The nanoparticles looked overall uniformly distributed and small aggregates of about 3 μ m were present throughout the matrix.

The collected scaffolds were further analysed by means of Energy-dispersive X-ray spectroscopy (EDX) to confirm the composition and the distribution of nanoparticles throughout the matrix, discriminating from inorganic particles and potential material beads.

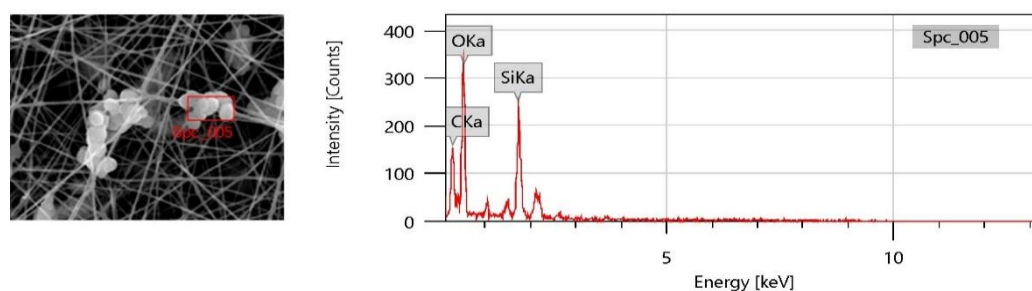


Figure 6. 9 EDS analysis performed on 25% COL/MBG_SG_Sr4% scaffolds.

As represented in Figure 6.9, EDX spectrum of the 25%COL/MBG_SG_Sr4% scaffold revealed the presence of MBG_SG_Sr4% particles, evidencing the contribution of silica by Si and O peaks, whereas the peak of C clearly arose from the collagenous matrix.

The reported results were supported and confirmed by ATR-FTIR analysis, where the preservation of the collagen physico-chemical features was also investigated by comparing the spectrum of original collagen powders with the

curves obtained from 25% COL and 25% COL/MBG_SG_Sr4% scaffolds, as represented in Figure 6.10.

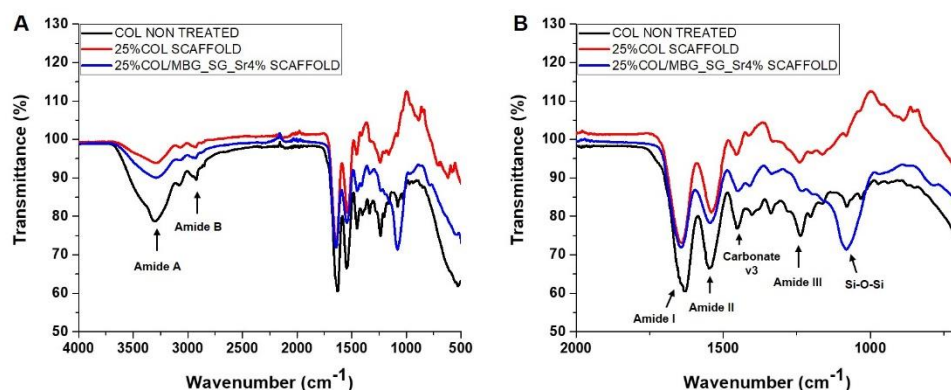


Figure 6. 10 ATR-FTIR of 25% COL and 25% COL/MBG_SG_Sr4% scaffolds compared to native collagen (non-treated): whole spectrum (A) and focus between 2000 and 700 cm^{-1} (B).

The spectrum of non-treated collagen powders displayed the characteristic peaks at 3305 cm^{-1} and 2922 cm^{-1} related to amide A and B bands respectively, while peaks at 1628 cm^{-1} , 1548 cm^{-1} and 1237 cm^{-1} were attributed to the amide I, amide II and amide II transmittance peaks [5,6] .

As summarised in Table 6.4, the spectra of 25% COL and 25% COL/MBG_SG_Sr4% scaffolds depicted similar patterns when compared with original collagen, without significant alterations in peak intensity or shifts. The slight shift of amide I in 25% COL and 25% COL/MBG_SG_Sr4% scaffolds was ascribed to potential residuals of acetic acid, normally detected at about 1700 cm^{-1} . However, the absence of an evident peak at 1700 cm^{-1} suggested an almost total evaporation of the solvent during the electrospinning process [2].

According to previous studies [1,2,4], significant shifts of amide I and II towards lower wavelength as well as the loss of amide III peak at about 1240 cm^{-1} indicate the presence of unfolded structures related to the partial or total denaturation of the protein. Therefore, the detection of amide III peak in both 25% COL and 25% COL/MBG_SG_Sr4% spectra as well as the absence of significant shifts for amide I and II, suggested the good preservation of the integrity of collagen triple helices structures. In detail, 25% COL/MBG_SG_Sr4% presented a weaker band for amide III at 1231 cm^{-1} due to the contribution of Si-O-Si band centred at about 1082 cm^{-1} of MBG_SG_Sr4% nanoparticles. The similar values registered for the different spectra also indicated that the addition of MBG_SG_Sr4% and the applied conditions did not destabilize the secondary structure of collagen.

The characteristic bands of the carbonate group corresponding to the ν_3 vibration of C-O was also registered at about 1450 cm^{-1} for all the samples.

Table 6. 4 ATR-FTIR characteristic peaks of collagen, 25% COL and 25%COL/MBG_SG_Sr4% scaffolds.

	Amide A	Amide B	Amide I (C=O stretching)	Amide II (NH stretching)	Carbonate ν_3	Amide III (CN bending)	Si-O-Si
COL non-treated	3305 cm^{-1}	2922 cm^{-1}	1628 cm^{-1}	1548 cm^{-1}	1453 cm^{-1}	1237 cm^{-1}	-
25% COL scaffold	3288 cm^{-1}	2930 cm^{-1}	1640 cm^{-1}	1540 cm^{-1}	1456 cm^{-1}	1239 cm^{-1}	-
25% COL/MBG_SG_Sr4% scaffold	3288 cm^{-1}	2932 cm^{-1}	1643 cm^{-1}	1544 cm^{-1}	1450 cm^{-1}	1231 cm^{-1}	1082 cm^{-1}

The characterisation performed on the developed electrospun composite scaffolds evidenced the successful achievement of homogeneous matrices reproducing the architectural feature of the native extracellular matrix of bone at the nanoscale. Moreover, the developed strategies proved to have less impact on the physico-chemical properties of type I collagen, preserving the supramolecular structure, in contrast with other studies reported in the literature.

Based on the promising results obtained, the increase of the inorganic phase concentration and the use of chemical crosslinking agents to obtain higher stability and mechanical strength of the final scaffolds, is currently under investigation.

6.4 Final considerations

The outcomes outlined in this Chapter confirmed the processability of collagen-based formulations for the design of biomimetic nanostructures scaffolds by means electrospinning technologies, highlighting the importance of a comprehensive assessment of both material chemistry and process conditions to obtain homogeneous systems.

One of the main advantages of electrospinning as additive manufacturing technique is represented by its potential scalability, where the process can be conducted in a laboratory setting or scaled up for larger scale production after few modifications [3]. On the other hand, the processing of different biopolymers with this technique has often demonstrated several complications due to the use of toxic and harsh solvents exploited to ensure the high processability of the systems, that in contrast, compromise the physico-chemical properties of the materials and its cytocompatibility [9].

Up to now, several conflicting results have been reported in the literature when type I collagen was used as main polymeric matrix to produce 3D scaffolds by means of electrospinning [4]. Despite different studies evidenced the loss of the main structural features of collagen (i.e. fibrils, triple helix, single helices) due to the harsh solvents and process conditions used, electrospun collagen scaffolds

demonstrated a positive biological response and cell attachment, higher than gelatine constructs [4].

The proper selection of solvents and electrospinning conditions was proved to be crucial for the preservation of the physico-chemical properties of collagen as well as the final morphological features of the scaffolds. Moreover, the original source of collagen powders and the different methods used for extractions from native tissues showed to significantly influence the final features of the constructs, justifying the contrasting results reported in the literature [4,10,11].

In this frame, 40% acetic acid solution was used to dissolve high concentrations of bovine collagen powders (25 wt%) to obtain a final system able to be processed by means of electrospinning and forming homogeneous fibrous mats with fibres measuring about 100 nm in diameter.

Previous tests using a mixture of acetic acid (AA), ethyl acetate (EA) and distilled water (dH₂O) with a ratio of 4:3:3 and lower concentrations of collagen (12 wt% and 20 wt%), showed poor stability of the solution and led to the formation of less homogeneous fibrous scaffolds presenting a great number of defects.

The electrospinning of collagen thus evidenced the promising advantage of replicating the nano- and micro-architecture of the bone tissue that is characterized by collagen fibrils ranging between tens of nanometers up to few microns [12,13].

Alongside the morphological aspects, the introduction of bioactive reinforcing inorganic phases was exploited to boost the regenerative effect of the final scaffolds. Based on the well-known bioactive character of mesoporous bioactive glasses and the limited data in the literature presenting their combination with collagen for electrospinning applications, MBG_SG_Sr4% (5 wt%) were successfully incorporated into the collagen system previously optimized and composed of 25 wt% collagen dissolved in 40% acetic acid.

After optimization of the process conditions, the resulting scaffolds presented a uniform nano-fibrous scaffolds and the homogeneous distribution of inorganic particles throughout the collagen matrix, while the further characterization of the constructs confirmed the preservation of the physico-chemical properties of collagen.

Since the manufacturing process does not promote the natural self-assembly and crosslinking of collagen molecules, crosslinking strategies are required to increase the stability and the final strength of the resulting electrospun scaffolds.

According to the goals of the European project GIOTTO and based on the results reported, higher concentrations of inorganic particles and different chemical treatments are currently under investigation in collaboration with the MERLN Institute, to design stable bioactive and nanostructured mats able to promote the regeneration of bone tissue for pelvic fractures.

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Conclusions and future perspectives

The aim of the present PhD research activities was the design of biomimetic collagen-based scaffolds for bone tissue regeneration, developed by exploring the use of type I collagen in combination with bioactive inorganic phases, i.e., strontium containing mesoporous bioactive glasses (Sr-MBGs) and nanohydroxyapatite (nano-HA), and its processing by means of additive manufacturing and electrospinning technologies. The study enabled the detection of advantages and limitations in the use of type I collagen as biomaterial for bone tissue engineering, as well as the optimisation of strategies to improve the stability and the processability of collagen-based materials, to finally obtain complex biomimetic constructs.

The research activities presented from Chapter 3 to Chapter 5, framed in the ERC BOOST Project (Biomimetic trick to re-balance Osteoblast-Osteoclast loop in osteoporosis treatment: a Topological and materials driven approach; ERC Consolidator Grant 2015, GA 681798), showed the development and the comprehensive characterisation of collagen-based bioactive biomaterial inks, alongside the assessment of different printing strategies and the resulting features of 3D scaffolds. Moreover, the cytocompatibility of the different developed systems and the preliminary biological assessment of the composite scaffolds were largely discussed.

As first objective, reported in Chapter 3, the combination of type I collagen with Sr-MBGs and nano-HA, successfully led to the creation of homogeneous systems able to reconstitute fibrous matrices embedding the inorganic phase nanoparticles upon physiological conditions (pH 7.4, 37 °C), partially resembling the composite nature and nano-architecture of bone tissue. Moreover, the addition of Sr-MBGs contributed to the development of bioactive systems, able to promote the deposition of calcium phosphate crystals in physiological fluids, as well as the release of pro-osteogenic strontium ions.

Starting from these promising results, future activities may aim at enhancing the bioactive character of the developed hybrid formulations by the introduction of additional biochemical signals such as growth factors, to boost the regenerative potential of the final systems. In this frame, some research activities conducted by the IRIS research group recently reported the use of smart nanocarriers to modulate the release of bioactive molecules in combination with 3D printed collagen matrices [1].

A second fundamental aspect to be addressed considered the poor mechanical strength and stability of the developed collagen-based materials, as already widely

reported in the literature [2,3]. Section 3.5 of Chapter 3 described the successful chemical crosslinking of all the collagen-based composites, exploiting a chemical treatment with genipin, able to promote the formation of covalent bonds between collagen molecules. The genipin crosslinking showed superior ability to enhance the stiffness and the resistance to both hydrolytic and enzymatic degradation of the collagen-based composites, compared to (1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) combined with N-hydroxysuccinimide (NHS) and 4-arm polyethylene glycol succinimidyl glutarate (4-StarPEG). Moreover, the use of ethanol as genipin solvent, instead of aqueous saline solutions, hindered the premature release of strontium ions during the crosslinking treatment. Based on these results, 0.5% genipin in 70% EtOH solution (GEN/EtOH) was selected as most efficient crosslinking treatment.

In particular, all the explored chemical strategies were developed in view of the further stabilisation of the scaffolds designed by means of extrusion printing, therefore considering the possibility to incubate the resulting constructs in a crosslinking medium for the required amount of time.

In this study, the evident increase of the mechanical stiffness and stability of the collagenous systems was thus obtained mainly exploiting the effect of the chemical crosslinker, whereas the addition of the inorganic particles into the collagen phase did not prove to significantly enhance the strength of the resulting composite. However, as future perspective, the reinforcing effect of the inorganic phases, both Sr-MBG and nano-HA, may be boost upon proper functionalisation of the particles, with the aim to promote the formation of covalent bonds with collagen molecules, thus creating effective hybrid systems [4].

A consistent part of the work was dedicated to a comprehensive rheological characterisation of the different collagen-based systems, described in Chapter 3, where the outcomes were exploited to preliminary assess the material printability.

In details, the flow ramps performed on the suspensions at 10 °C evidenced their marked shear thinning behaviour, confirming the extrudability even at high shear rates. However, the low values of viscosity, as well as the sol-gel transition kinetics occurring at 37 °C, suggested the need to support the deposition of the soft biomaterial inks during the printing process.

Based on the rheological assessment and the physico-chemical characterisation of the designed systems, the results presented in Chapter 4 described the development of strategies for the extrusion printing of the soft biomaterial inks.

In this frame, the freeform reversible embedding of suspended hydrogel (FRESH) method demonstrated its suitability for the extrusion printing of collagen-based formulations, leading to the design of high-resolution mesh-like structures upon previous optimisation of the printing parameters. Higher printing fidelity and scaffold stability was obtained improving the morphological and rheological features of gelatine slurries exploited as supporting baths [5]. Despite the high printing fidelity achieved, the poor visco-elastic properties of the composite

scaffolds after incubation at 37 °C, led to the partial collapse of the structure upon gelatine removal, hindering the realisation of more complex architectures.

Based on the recent study reported by Lee and co-workers [6], future experiments will be focused on the use of acidic suspensions containing higher collagen concentrations, where the buffering ability of the improved gelatine slurry (FRESH V2) will be exploited to promote the instantaneous gelation of the deposited filaments and thus the previous stabilisation of the printed constructs.

Despite the constraints in achieving highly complex architectures, the developed printing strategies proved the ability to achieve mesh-like and honeycomb patterns with high resolution, where the precision of the resulting structures were not compromised by the subsequent chemical crosslinking with genipin.

Moreover, with the aim to increase the stability of the deposited material before the removal of the supporting bath, an alternative strategy for the in-situ crosslinking of the collagen-based systems is currently under investigation. In details, this method considers the addition of genipin directly into the material formulation, where an accurate assessment of the most suitable chemical crosslinker concentration is required in order to preserve the printability and stability of the biomaterial ink, alongside its final cytocompatibility.

With the aim to address the limitations presented by the previous printing strategies, and in particular the soft nature of the resulting scaffolds before chemical crosslinking, Chapter 4 additionally presented the development of hybrid photo-curable formulations based on methacrylated collagen, Sr-MBGs and nano-HA. These hybrid formulations were developed to enable the design of more complex architectures, by exploiting the UV crosslinking of the scaffold layer-by-layer. The high visco-elastic properties, the extrudability and the fast crosslinking kinetics of the developed materials were proved by means of the rheological assessment alongside printing tests, performed following the previously optimised FRESH method.

These preliminary results are currently exploited to design stable bone-like scaffolds, considering the simultaneous deposition of biomaterial ink and supporting material. To this aim, STL files closely reproducing the trabecular structure of bone were developed and optimised, demonstrating their processability with the commercial BIOX Bioprinter.

Thanks to the experiments performed in collaboration with Istituto Ortopedico Rizzoli of Bologna (Scienze e Tecnologie Biomediche, IRCCS) and Università Politecnica delle Marche (Department of Clinical and Molecular Sciences – DISCLIMO), the cytocompatibility of the developed formulations and crosslinking methods was confirmed by using pre-osteoblastic MG-63 and Saos-2 cell lines.

The biological assessment additionally confirmed the high potential of osteoblast (OB) and osteoclast (OC) co-cultures for the pre-screening of new materials and scaffolds.

In particular, the human OB/OC co-culture model developed by Istituto Ortopedico Rizzoli successfully confirmed the ability of both Sr-MBG and nano-HA containing collagenous systems to promote cell adhesion, proliferation, and differentiation. Despite the promising results, further experiments and more detailed analyses are required to assess the specific influence of the different bioactive components over cell behaviour and cross-talks, particularly focusing on genetic expression and the detection of additional differentiation markers.

The high biocompatibility, the physico-chemical properties and the great printability demonstrated by the developed collagen-based systems, pave the way for their potential application as biomaterial inks for the fabrication of biomimetic bone models. In this context, the different strategies optimised during this research study and the further combination of the developed bioactive materials with different cells and bioactive molecules, may lead to the design of viable biomimetic constructs reproducing reliable bone models [7].

Finally, based on the main goals of the present PhD study, the potential design of biomimetic collagen-based scaffolds by means of electrospinning technologies was reported in Chapter 6.

The research activities were conducted in the frame of the GIOTTO European Project (Active aGeIng and Osteoporosis: The next challenge for smart nanobiOmaterials and 3D technologies; NMBP-22-2018, GA 814410) and performed in collaboration with the Institute for Technology-Inspired Regenerative Medicine (MERLN) under the supervision of Prof. Lorenzo Moroni.

In contrast to different studies reported in the literature [8], the preservation of the physico-chemical and structural properties of type I collagen was demonstrated upon the proper assessment of solvents and protein concentrations.

Moreover, the optimisation of the electrospinning parameters led to the formation of nanometric fibrous scaffolds, highly resembling the morphological features of bone extracellular matrix at the nanoscale. Moreover, the developed collagen formulation demonstrated its suitability for the incorporation of Sr-MBG nanoparticles and the subsequent fabrication of nanocomposite fibrous scaffolds.

In accordance with the objectives of the GIOTTO project, these preliminary results are currently exploited in the consortium to design more complex and stable scaffolds combining different bioactive inorganic phases and collagen sources. Future studies will be also focused on the opportunity to obtain more complex architectures exploiting the direct writing electrospinning method, while confirming the high bioactive features of the constructs by means of a comprehensive biological assessment.

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Posso finalmente dire che sono riuscita a oltrepassare questa barriera.

In fondo, quando il gioco si fa duro, i duri cominciano a giocare!