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A new bi-layered scaffold for osteochondral tissue regeneration: *In vitro* and *in vivo* preclinical investigations

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ABSTRACT

Current treatments for acute or degenerative chondral and osteochondral lesions are in need of improvement, as these types of injuries lead to disability and worsen the quality of life in a high percentage of patients. The aim of this study was to develop a new bi-layered scaffold for osteochondral tissue regeneration through a "*biomimetic*" and "*bioinspired*" approach. For chondral regeneration, the scaffold was realized with an organic compound (type I collagen), while for the regeneration of the subchondral layer, bioactive magnesium-doped hydroxyapatite (Mg/HA) crystals were co-precipitated with the organic component of the scaffold. The entire scaffold structure was stabilized with a cross-linking agent, highly reactive bis-epoxyde (1,4-butanediol diglycidyl ether – BDDGE 1 wt%). The developed scaffold was then characterized for its physico-chemical characteristics. Its structure and adhesion strength between the integrated layers were investigated. At the same time, *in vitro* cell culture studies were carried out to examine the ability of chondral and bone scaffold layers to separately support adhesion, proliferation and differentiation of human mesenchymal stem cells (hMSCs) into chondrocytes and osteoblasts, respectively. Moreover, an *in vivo* study with nude mice, transplanted with osteochondral scaffolds plain or engineered with undifferentiated hMSCs, was also set up with 4 and 8-week time points. The results showed that chondral and bone scaffold layers represented biocompatible scaffolds able to sustain hMSCs attachment and proliferation. Moreover, the association of scaffold stimuli and differentiation medium, induced hMSCs chondrogenic and osteogenic differentiation and deposition of extracellular matrix (ECM). The ectopic implantation of the engineered osteochondral scaffolds indicated that hMSCs were able to colonize the osteochondral scaffold in depth. The scaffold appeared permissive to tissue growth and penetration, ensuring the diffusion of nutrients and oxygen, as also suggested by the presence of a neo-angiogenesis process, especially at 4 weeks. Moreover, the *in vivo* results further confirmed the great potential of the scaffold in tissue engineering, as it was able to support the initial formation of new bone and chondral tissue, confirming the importance of combined and innovative strategies to improve the available therapeutic strategies for chondral and osteochondral regeneration.

1. Introduction

Traumatic and degenerative chondral and osteochondral lesions represent a public health problem related to the worldwide increase of the aging population, unhealthy lifestyles and sport injuries, which largely lead to disability and worsen patients' quality of life [1]. These conditions affect an ever-increasing number of people with huge socio-economic impacts. The main clinical studies reported in literature up to date suggest that 20–60% of knee arthroscopic procedures reveal focal chondral or osteochondral defects when assessed by the ICRS evaluation criteria [2]. In 2004 Arøen A. et al., reported the results of 993 arthroscopies performed in young patients (mean age 35 years) finding that 66% of patients were affected by cartilage pathology, and an ICRS grade III and IV characterized 11% of patients [3]. In a retrospective study on 25,124 knee arthroscopies, focal chondral or osteochondral lesions have been found in 67% of patients with joint pain [4]. If left untreated, they lead to the progression of symptoms (pain, dysfunction and joint degeneration) up to osteoarthritis, which in fact represents the fourth most common cause of hospitalization [5]. Even though several therapeutic approaches have

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been developed to treat these defects, none of them has yet proved to be quite successful, as they all have drawbacks and limitations and often do not provide sufficient tissue regeneration and recovery to original functions [6,7]. In 2013, 92% of total procedures was represented by short-term pain relief treatments, such as debridement/cartilage shaving (69%), or by unsatisfactory solutions, such as microfractures (23%) [8,9].

For these reasons, there is a growing demand of new strategies able to promote tissue regeneration of osteochondral defects: a difficult goal to achieve given the dual composition of cartilage and subchondral bone with intrinsic biological, biochemical, and biomechanical differences and properties [10]. These markedly different properties challenge the integration and stability of the newly regenerated tissue at the cartilage–bone (osteochondral) interface. Thus the use of a scaffold with an integrated architecture that can support both bone and cartilage tissues is paramount to achieve successful osteochondral regeneration. A variety of different materials: natural or synthetic, polymeric or ceramic, but also a combination of these, have been studied to develop scaffolds able to exhibit osteochondral inductivity and conductivity, mimicking the matrices of both tissues [11]. Fiber orientation, porosity, pore size and interconnectivity, together with wettability of the scaffold surfaces, regulate cellular attachment and infiltration of the matrix, thereby tuning the regeneration process. Synthetic polymers offer an attractive opportunity but may have limitations in bioactivity because of their hydrophobic surface [12]. Natural polymers, such as collagen, are mechanically weaker, but they are flexible to adapt to the joint curvature in the treatment of large defect, and they usually contain specific molecular domains that induce and support cell bioactivity. One of the most promising strategies is represented by the realization of a bi-layered scaffold obtained by well-connected gradient of natural materials. This encourages the infiltration of cells from the bone marrow through the scaffold itself and guides the regeneration of layer-specific chondral or bone matrix [13,14]. Moreover, a stable transition region at the interface would prevent scaffold delamination after implantation.

The aim of this study was the biological assessment of a bi-layered scaffold created to improve the regeneration of both bone and cartilage, taking into account their various biological features. Both scaffold components were synthesized with type I collagen for chondral regeneration, and co-precipitated with bioactive magnesium-doped hydroxyapatite (Mg/HA) crystals in the subchondral layer for bone regeneration.

It is well known that synthetic HA represents a widespread material in the orthopedic application because of its structural similarity to the inorganic phase of bone tissue. In particular, the substitution of calcium with small amount of biologically active ions (*i.e.*, Mg^{2+}) in nonstoichiometric nanocrystalline apatites, has been developed to overcome the traditional graft material disadvantages [15] and to ameliorate its physico-chemical, structural and morphological features.

Mg^{2+} also plays a critical role in the bone formation process, being involved in the early stage of osteogenesis and bone formation. The obtained MgHA/Coll hybrid composite seems to better mimic the matrix of newly formed bone, and to increase *in vivo* osteoconductivity compared to commercial stoichiometric HA granulate [16,17].

Scaffold microstructure and the ability of the material to swell in physiological environment, without compromising the scaffold integrity even after cyclic testing, have been verified. Subsequently, *in vitro* cell culture studies were carried out to evaluate the ability of chondral and bone mono-layered scaffold to support growth and differentiation of human mesenchymal stem cells (hMSCs) into chondrocytes and osteoblasts, respectively. The bi-layered scaffold was later tested *in vivo* by using a heterotopic implantation site in an immunocompromised mouse model.

2. Materials and methods

2.1. Scaffold design and characterization

2.1.1. Synthesis of the bi-layered scaffolds

The scaffolds for *in vitro* (bi-layered scaffolds for physical-chemical characterizations: ∅ = 10–12 mm, h = 5 mm; and mono-layered scaffolds for cell cultures: ∅ = 7 mm, h = 5 mm) and *in vivo* (bi-layered scaffolds ∅ = 2 mm, h = 3 mm) studies were manufactured by Fin-Ceramica Faenza SpA (Faenza – Ravenna, Italy). Two different mono-layered scaffolds - chondrogenic (C) and osteogenic (O) layers- were manufactured, and a bi-layered scaffold was produced by combining the layers in a volumetric ratio of 1: 2 (O:C). For the C layer, an aqueous acetic buffer solution (pH = 3.5) of type I atelocollagen (1 wt%) supplied as an acetic gel, was diluted with highly purified water (milli-Q) and subsequently precipitated by dropping 0.1 M NaOH solution to reach the isoelectric point (pH = 5.5). For the O layer, a 0.04 M H_3PO_4 solution was mixed with the aqueous acetic buffer solution of type I atelocollagen (1 wt%). The obtained solution was dropped into a basic suspension containing $Ca(OH)_2$ 0.04 M, $MgCl_2\ 6H_2O$ (2×10^{-3} M) and Simulated Body Fluid (SBF) to yield a Magnesium-HA/Collagen material with a theoretical 70/30% ratio and a Mg/Ca molar ratio of 5% in the crystal lattice. For both layers, the obtained precipitate fibers were maturated for 1 h and then washed with highly purified water.

2.1.2. Material crosslinking

Both C and O layers, as well as the bi-layered scaffolds, underwent a crosslinking treatment by 48 h immersion in an aqueous solution of 1,4-butanediol diglycidyl ether (BDDGE) crosslinking agent. Based on previous work [18], the chosen parameters were as follows: 1 wt% BDDGE, 37 °C temperature, and $NaHCO_3/Na_2CO_3$ buffer solution at pH = 9.5. After the cross-linking reaction, both layers underwent a freeze-drying treatment consisting in a controlled freezing/heating ramp (from 25 °C to −35 °C, from −35 °C to 20 °C), carried out over 25 h under vacuum conditions (0.29 mbar) to consolidate the 3D scaffold. Finally, the scaffolds were packed separately and sterilized with γ radiation at 25 kGy.

2.1.3. Physical-chemical characterizations

Details and results of the physical-chemical characterization of C and O layers have been previously reported [18]. EDS and TEM observations have been performed and described in Supplementary Materials.

The adhesion force between C and O layers was measured by t-peel test by tension loading on bi-layered scaffolds. It was determined using a custom designed sample holder compatible with a universal MTS machine of 7tf with a cell load of 100 N. Each scaffold was attached to the holder using cyanoacrylate glue, and its superficial penetration was verified before starting the test. Tests were conducted with a constant traction speed of 3 mm/min up to failure. The interfacial adhesion force was defined as the highest registered load in the force–displacement curve. The interfacial adhesion strength was calculated using the adhesion force at failure, divided by the sample area.

The bi-layered scaffold swelling capacity was then evaluated using dry scaffolds, which were soaked at room temperature (RT) in PBS solution for 5 min, reaching a stabilization of the scaffold dimension. Scaffolds were weighed and their diameter and thickness were measured before and after swelling. Swelling was then determined as percent

Table 1
Primer sequences of the gene investigated for chondrogenic and osteogenic differentiation.

Gene	Forward Primer	Reverse Primer
Chondral differentiation		
SOX9	GAGCAGACGCACATCTC	CCTGGGATTGCCCCGA
ACAN	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
COL2A1	GTTTTCCAGCTTCACCATCATC	CCTCAAGGATTTCAAGGCAAT
Bone differentiation		
RUNX2	QuantiTect Primer Assay (Qiagen)	Hs_RUNX2_1_SG
ALPL	QuantiTect Primer Assay (Qiagen)	Hs_ALPL_1_SG
COL1A1	QuantiTect Primer Assay (Qiagen)	Hs_COL1A1_1_SG
SP7	QuantiTect Primer Assay (Qiagen)	Hs_SP7_1_SG
BGLAP	ACACTCCTCGCCCTATTG	GATGTGGTCAGCCAACTC
Reference gene		
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA