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Ribose mediated crosslinking of collagen-hydroxyapatite hybrid scaffolds for bone tissue regeneration using biomimetic strategies

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ABSTRACT

This study explores for the first time the application of ribose as a highly biocompatible agent for the crosslinking of hybrid mineralized constructs, obtained by bio-inspired mineralization of self-assembling Type I collagen matrix with magnesium-doped-hydroxyapatite nanophase, towards a biomimetic mineralized 3D scaffolds (MgHA/Coll) with excellent compositional and structural mimicry of bone tissue. To this aim, two different crosslinking mechanisms in terms of pre-ribose glycation (before freeze drying) and post-ribose glycation (after freeze drying) were investigated. The obtained results explicate that with controlled freeze-drying, highly anisotropic porous structures with opportune macro-micro porosity are obtained. The physical-chemical features of the scaffolds characterized by XRD, FTIR, ICP and TGA demonstrated structural mimicry analogous to the native bone. The influence of ribose greatly assisted in decreasing solubility and increased enzymatic resistivity of the scaffolds. In addition, enhanced mechanical behaviour in response to compressive forces was achieved. Preliminary cell culture experiments reported good cytocompatibility with extensive cell adhesion, proliferation and colonization. Overall, scaffolds developed by pre-ribose glycation process are preferred, as the related crosslinking technique is more facile and robust to obtain functional scaffolds. As a proof of concept, we have demonstrated that ribose crosslinking is cost-effective, safe and functionally effective. This study also offers new insights and opportunities in developing promising scaffolds for bone tissue engineering.

1. Introduction

Over the last decades, the idea of bone tissue engineering (BTE) has gained significant interest leading to the development of a plethora of biomaterials with suitable biocompatibility, competent micro-architecture, controlled degradability and enhanced osteoconductive properties [1–3]. Currently, identifying the key osteogenic cues and adopting the biomimicry strategy is of greater importance in order to obtain hierarchically organized structures similar to the anatomical bone with the ability to activate mechano-transduction processes and to yield regeneration of well-organized bone [4]. In bone, the extracellular matrix (ECM) consists of organic (35 wt%) and inorganic (65 wt%) phases, where the predominant organic protein, collagen, drives the heterogeneous nucleation of hydroxyapatite (HA) nano-crystals onto its fibrils, by activation of specific control mechanisms [5], thus generating a mineralized structural unit of bone characterized by high resilience and compressive strength [6,7]. Developing biomimetic scaffolds by bio-inspired strategies has been a successful approach with proven results. For example, bone-like hybrid composites obtained through the direct nucleation of HA nano-crystals on self-assembling collagen fibrils showed structural, compositional and morphological similarities to the natural bone and biomimetic osteochondral composites were also developed with differentially ability to support cartilage and bone tissue regeneration [8,9]. The close mimicry of the composition and crystallinity of the bone mineral has shown significant positive effect on bone formation and remodelling [10]. In particular, magnesium (Mg^{2+}) is a bivalent ion largely present in the young or newly formed bone. The substitution of calcium by magnesium in the surface crystal site increases the chemical-

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physical likeness of HA nanoparticles to biologic HA eventually with a beneficial impact on protein adsorption and cell adhesion to the scaffolds. The incorporation of Mg^{2+} ions in the HA lattice has also highly improved the osteoconductive activity with enhanced bone repair in *in-vivo* studies [11,12].

The recent advances in material science offer the innovative concept of macromolecular crosslinking which enables the fine tuning of hybrid constructs in terms of mechanical and bio-resorption properties. Accordingly, numerous crosslinking agents such as glutaraldehyde (GA), dehydrothermal (DHT), 1,4-butanediol diglycidyl ether (BDDGE), 1-ethyl-3-(3-dimethylamino propyl), carbodiimide hydrochloride (EDC) and genipin offer significant advantages in developing mechanical stable scaffolds [13]. This apart, ribose glycation is a simple, natural non-enzymatic crosslinking reaction, where the reducing sugar interacts with the amino group of proteins to create chemical alteration in their structure which is known as Schiff's base alteration. This Schiff's base rearranges to form an Amadori product, which allows the protein-to-protein crosslinking to occur and eventually leads to the formation of advanced glycation products (AGEs) [14,15]. The formation of AGEs are responsible for the increased matrix stiffness, decreased solubility and high enzymatic resistivity of the crosslinked tissue [16–18]. Adopting this interesting glycation technique under *in-vitro* and *in-vivo* conditions is widely investigated nowadays to improve tissue functionality. Accordingly, several, *in-vitro* studies have demonstrated that ribose glycation has a significant role in modifying the mechanical properties of bone [19–21]. In particular, studies have shown that minimal concentration of ribose (30 mM) has a significant effect on intermolecular crosslinking of collagen [22]. Similarly, glucose-modified gelatin/collagen matrix, demonstrated to be a safe and effective biomaterial with excellent biocompatibility [23]. Besides this, *in-vivo* studies have successfully used ribose-crosslinked-collagen as dermal fillers in rabbit model, where the results demonstrated increased functional longevity with better soft-tissue augmentation [24]. In addition, the clinical evidence also suggests that ribose crosslinking of collagen is safe and effective in supporting guided dental bone regeneration [25,26]. Therefore, in this study we investigated the feasibility in developing novel bone-like scaffolds by bio-inspired, pH-driven, mineralization of type I collagen matrix with magnesium-doped hydroxyapatite nanophase (MgHA/Coll). The physical stability of the obtained MgHA/Coll hybrid composites was enhanced by using ribose crosslinking in two different approaches (pre and post glycation) and the resultant scaffolds were characterized physico-chemically. The cell-material interaction was evaluated using mouse pre-osteoblast (MC3T3-E1) cells under *in-vitro* conditions which showed favourable cell adhesion and proliferation. To best of our knowledge, this is the first study investigating the effect of ribose glycation as crosslinking agent in biomineralized collagen matrices.

2. Experimental section

2.1. Materials

Type I collagen from equine tendon (1 wt% in acetic buffered solution (pH 3.5) was supplied by Opocrin SpA, (Italy) and was used as organic matrix to synthesis MgHA/Coll hybrid composite. Phosphoric acid (H_3PO_4, purity 85 wt%), calcium hydroxide ($Ca(OH)_2$, purity 95 wt%) and magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, purity 99 wt%), Ribose ($C_5H_{10}O_5$, purity 99 wt%), ethanol (C_2H_6O, purity 95wt%), bacterial collagenase enzyme from (*Clostridium histolyticum*), TritonX-100, glutaraldehyde (grade II) and FITC-conjugated Phalloidin were purchased from Sigma Aldrich, (USA). Trypsin-EDTA, α-MEM (Minimal essential media), penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco, (USA). Live/Dead assay kit and DAPI stain were purchased from Invitrogen, (USA). Phosphate buffer saline (PBS, pH 7.4), was purchased from EuroClone (Italy).

2.2. Synthesis of MgHA/Coll hybrid composite

Briefly, two aqueous suspensions were prepared: (i) acidic suspension: 2.4 g of H_3PO_4 was diluted with 500 ml of milli-Q water and added to 150 g of 1 wt% collagen gel to obtain an aqueous homogenous suspension (pH 2.5). (ii) basic suspension: 2.7 g of $Ca(OH)_2$ and 0.35 g of $MgCl_2 \cdot 6H_2O$ were dispersed in 500 ml of milli-Q water to obtain an aqueous homogenous suspension (pH 12.0). Later, the acidic suspension was added drop-wise to the basic suspension at 25 °C under continuous stirring. The biomineralization process is a slow pH-driven process (decrease in pH from 12.0 to 7.5), where the neutralization reaction causes the MgHA nanocrystals formation on collagen fibres and the simultaneous self-assembling of collagen fibrils with the consequent precipitation of the hybrid composite MgHA/Coll. The reaction product was kept at static condition for 2 h at 25 °C to mature. After the biomineralization process, the composite slurry was washed three times in milli-Q water and filtered through metallic sieve (150 μm) to eliminate any unreact product and counterions. The final recovered composite slurry is referred as MgHA/Coll and was used to produce ribose crosslinked scaffolds.

2.3. Ribose glycation of MgHA/Coll scaffolds

In this study two different crosslinking strategies were employed to investigate the effect of ribose glycation in three-dimensional (3D) MgHA/Coll scaffolds which as follows,

(i) Pre-glycation: Biomineralized MgHA/Coll recovered as slurry was crosslinked in ethanol and PBS solution (70:30 vol.%) with 30 mM of ribose under intermittent shaking condition at 37 °C for 5 days. Then, the slurry was washed in milli-Q water three times to remove unbound ribose and residual solvents. Later the slurry was filled onto a polystyrene well-plate and freeze-dried by freezing at −40 °C and drying at 25 °C (5 Pascal, LIO 3000 PLT, Italy) for 48 h under a constant vacuum of 0.1 mbar to obtain 3D porous scaffolds and hereafter referred as MgHA/$Coll_{Pre}$ scaffolds.

(ii) Post-glycation: Biomineralized MgHA/Coll recovered as slurry was filled onto a polystyrene well-plate and freeze-dried using the same above-mentioned conditions. Thus, obtain 3D porous scaffolds were crosslinked in ethanol and PBS solution (70:30 vol.%) with 30 mM of ribose under intermittent shaking condition at 37 °C for 5 days. Later the scaffolds were washed in milli-Q water three times to remove unbound ribose and residual solvents and later freeze-dried again using the same above-mentioned conditions. The final 3D porous scaffolds were hereafter referred as MgHA/$Coll_{Post}$ scaffolds.

3D Porous non-crosslinked MgHA/Coll scaffolds served as reference material where ever needed in this study.

2.4. Physical-chemical characterization

Inductively coupled plasma-optical emission spectrometry (ICP-OES, Agilent Technologies 5100 ICP-OES, Santa Clara, USA) was used for the quantitative determination of Mg^{2+}, Ca^{2+} and PO_4^{3-} ions that constitutes the inorganic mineral component. Briefly, 40 mg of sample was dissolved with 2 ml nitric acid (65 wt%) followed by subsequent sonication and dilution with 100 ml of milli-Q water.

Absorption spectra in the infrared wavelength region were collected using Nicolet 380 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, USA). Briefly, 2 mg of sample was mixed with 100 mg of anhydrous potassium bromide and then pressed at 8000 psi into 7 mm diameter discs. The spectra were collected in the wavelength ranging from 400 to 4000 cm^{-1} with 2 cm^{-1} of resolution.