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Biomimetic fiber mesh scaffolds based on gelatin and hydroxyapatite nano-rods: Designing intrinsic skills to attain bone reparation abilities

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ABSTRACT

Intrinsic material skills have a deep effect on the mechanical and biological performance of bone substitutes, as well as on its associated biodegradation properties. In this work we have manipulated the preparation of collagenous derived fiber mesh frameworks to display a specific composition, morphology, open macroporosity, surface roughness and permeability characteristics. Next, the effect of the induced physicochemical attributes on the scaffold's mechanical behavior, bone bonding potential and biodegradability were evaluated. It was found that the scaffold microstructure, their inherent surface roughness, and the compression strength of the gelatin scaffolds can be modulated by the effect of the cross-linking agent and, essentially, by mimicking the nano-scale size of hydroxyapatite in natural bone. A clear effect of bioactive hydroxyapatite nano-rods on the scaffolds skills can be appreciated and it is greater than the effect of the cross-linking agent, offering a huge perspective for the upcoming progress of bone implant technology.

1. Introduction

The physical and biological requests of an ideal synthetic bone substitute involve frameworks that closely mimic human tissue morphology, but also that are optimized to perform host tissue's specific functions [1]. The human skeleton has an obvious mechanical function in supporting and protecting the body [2]. Therefore, it becomes critically important to design scaffolds that must maintain their physical integrity during applications subjected to mechanical stresses, which might lead to bearing superior body loads [3]. In a previous work [4], we have evaluated the hydrodynamic and crowding evolution of aqueous gelatin/hydroxyapatite nano-rods systems with the aim of increasing the knowledge about the collagen mineralization biomimesis, and how it can be manipulated for the preparation of collagenous derived frameworks with specific morphological characteristics. Gelatin is a partially degraded product of collagen; compared to its precursor has lower cost, it is low-antigenic, and retains specific collagen information signals, such as the Arginyl-glycyl-aspartic acid (RGD) tri-peptide, which can partially enhance the cell-adhesive activity [5,6]. Recently, gelatin-based biomaterials have been applied to artificial skin, [7] bone grafts, [8] and scaffolds for tissue engineering [9,10]. In this study, we have prepared improved gelatin-hydroxyapatite (HA) nano-composites using a natural phenolic compound, tannic acid (TA), as a cross-linking agent to reduce the solubility of gelatin in aqueous environments at the human-body temperature [5]. TA exhibits antioxidant activity, in addition of recently reported anti-allergenic, anti-inflammatory, anti-microbial, cardio-protective and anti-thrombotic properties [11] making this compound a very interesting raw material for the development of novel medical applications. Bio-mineralization, compression, bone bonding potential and degradation tests were carried out to determine the potential use of gelatin-HA scaffolds on calcified tissue reparation. It was found that the microstructure and the compression strength of the gelatin scaffolds can be modulated by the effect of the crosslinking agent and, particularly, by mimicking the nano-size scale of HA in natural bone. The presence of pre-embedded HA nanoparticles on gelatin scaffolds has a clear effect on the epitaxial growth of the mineralized phase deposition denoting their potential role in the process of

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bio-mineralization and, subsequently in the scaffolds' mechanical behavior, bone bonding potential and biodegradability properties.

2. Materials and methods

2.1. Reagents

Hexadecyl-trimethyl ammonium bromide (CTAB, MW = 364.48 g mol^{-1}, 99% Sigma-Aldrich), poly (propylene glycol) (PPG, Sigma-Aldrich, MW = 425 g mol^{-1}, δ = 1.004 g cm^{-3} at 25 °C), sodium phosphate (Na_3PO_4, MW = 148 g mol^{-1}, 96% Sigma-Aldrich), calcium chloride ($CaCl_2$, MW = 91 g mol^{-1}, 99% Sigma-Aldrich), sodium nitrite ($NaNO_2$, MW = 69 g mol^{-1}, 97% Sigma-Aldrich), acetic acid ($C_2H_4O_2$, MW = 60.05 g mol^{-1}, 99% Sigma-Aldrich), sodium acetate tri-hydrate ($C_2H_3NaO_2{\cdot}3H_2O$, MW = 136.03 g mol^{-1}, 99% Sigma-Aldrich), sodium hydroxide (NaOH, MW = 40 g mol^{-1}, 90% Sigma-Aldrich), commercial gelatin (GE) from bovine skin (Grade OR, Type B, 225 Bloom, MW ≈ 50000 g mol^{-1}, Merck), tannic acid ($C_{76}H_{52}O_{46}$, MW = 1701.20 g mol^{-1}, 99% Sigma-Aldrich), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 99% Sigma-Aldrich), phosphate buffer saline (PBS tablets, Sigma-Aldrich) and lysozyme from chicken egg (LSZ for molecular biology, Sigma-Aldrich) were used without further purification. For solutions preparation, only triplet-distilled water was used.

2.2. Preparation of fibrous gelatin-nano-HA scaffolds

Bone-like HA nanoparticles of 8 ± 1 nm diameters and 28 ± 3 nm length were made by a previously described methodology [12], for details see supplementary material (SM). To obtain the cross-linked gelatin-HA scaffolds, a 0.80 g/mL GE solution was prepared by dissolution of the proper amount of commercial GE in 35 mL of sodium acetate buffer (pH = 4.5). First, GE was left to hydrate for 30 min at RT, and then dissolved at 58 °C under stirring at 500 rpm during 30 min; finally it was allowed to rest in a thermostatic bath for 24 h in order to reach equilibrium. Afterward 0.85 mg/mL of HA nanoparticles were added to the previously described GE solution under vigorous sonication. After the homogeneous dispersion of HA into GE solution was obtained, cross-linking modified scaffolds were prepared using TA as cross-linking agent. First, GE-HA solution's pH was adjusted to pH = 11 by addition of $NaHCO_3$. Second, TA powder (12.4 and 33.3 mg/g GE) was added to the solution slowly under stirring at 500 rpm. Finally, after the integration of all reagents, the solution was magnetically stirred for 20 min to achieve the crosslinking process. The obtained gels were cooled under −50 °C during 24 h and lyophilized in a Rificor L-A-B4 lyophilizer. The selected amounts of GE, TA and HA used in this work are the suitable to guarantee an effective cross-linking effect on the gelatin matrix avoiding the grafting and branching reactions in conjunction with the hydrogen bonding between gelatin and TA molecules [13]; and for the attainment of an uniaxial orientation porous framework [4]. A summary of the reagent quantities was shown in supplementary material (Table SM1). Scaffolds mineralization process was performed following the layer by layer deposition method described by Taguchi et al. [14], see details in SM.

2.3. Structural characterization of cross-linked gelatin-HA scaffolds

2.3.1. Field emission scanning electron microscopy (FE-SEM)

Surface morphology was evaluated using a field emission scanning electron microscope (ZEISS FE-SEM ULTRA PLUS). To acquire all the SEM images a secondary electron detector was used. The accelerating voltage (EHT) applied was 3.00 kV with a resolution (WD) of 2.1 nm. Local compensation of charge (by injecting nitrogen gas) was applied avoiding the sample staining. The associated energy-dispersive spectrophotometer provided qualitative information about surface elemental composition. The pore sizes of scaffolds (determined from about 100 measurements) and the topography (calculating the Skewness (R_{sw}) and Kurtosis (R_{ku})) of samples were quantified from SEM microphotographs using an image visualization software (Image J 1.34 s, NIH Image, USA) [15] with an uncertainty of 5%. For details, see SM.

2.3.2. High resolution transmission electron microscopy (H-TEM)

H-TEM microphotographs were taken using a Libra 200 FE OMEGA transmission electron microscope operated at 200 kV with magnification of 1,000,000×. Observations were made in a bright field. Powdered samples were placed on carbon supports of 2000 mesh. The equipment is provided with electron diffraction (ED) system; d-spacing is computed based on the Bragg Law derived equation ($rd = L\lambda$), where "r" is the spot distance in the ED pattern, and $L\lambda = 1$ is the camera constant.

2.3.3. X-ray powder diffraction

Powder X-ray diffraction (XRD) data were collected with a Philips PW 1710 diffractometer with Cu Kα radiation (λ = 1.5418 nm) and graphite monochromator operated at 45 kV; 30 mA and 25 °C.

2.3.4. FT-IR spectroscopy

The experiments were done in a VARIAN FT-IR 670 spectrophotometer. To avoid co-adsorbed water, the samples were dried under vacuum until constant weight was attained and diluted with KBr powder before the FT-IR spectra were recorded.

2.4. Open porosity

The open porosity of the synthesized scaffolds was measured based on Archimedes's principle using a specific gravity bottle. We optimized this methodology by testing various immersion liquids as well as by establishing a standard test procedure following the Active Standard ASTM B962. Briefly, the open porosity of cubic shaped (≈ 0.71 cm edge) scaffold's samples was determined as follows: [16]

$$Porosity\,(\%) = \frac{(W_2 - W_3 - W_s)/\rho_e}{(W_1 - W_3)/\rho_e} \quad (1)$$

where, W_1 is the specific gravity bottle weight filled with ethanol, W_2 is the specific gravity bottle weight including ethanol and scaffold section, W_3 is the specific gravity bottle weight measured after taking out ethanol-saturated scaffold section from W_2, W_s is the ethanol-saturated scaffold section weight and ρ_e the density of ethanol; thus $(W_1 - W_3)/\rho_e$ is the total volume of the scaffold including pores and $(W_2 - W_3 - W_s)/\rho_e$ is the pore volume in the scaffold.

2.5. Swelling kinetics and solvent absorption capacity

Swelling kinetics of the samples were carried out following a conventional gravimetric procedure. [16] Vacuum-dried cubic scaffolds samples (0.71 cm edge) were weighed before and after soaking in 0.1 M PBS kept in a thermostatic water bath at 37 °C. Samples were taken out after regular intervals of time and weighted. Weight of all gels was taken until the equilibrium was reached. Each experiment was repeated three times. The water uptake capacity ($\%W_u$) is given by: [16]

$$\%W_u = \frac{(W_t - W_d)}{W_e} \times 100 \quad (2)$$